

**FACTORS INFLUENCING THE LIBERATION OF  
ACETYLCHOLINE AT CHOLINERGIC NERVE ENDINGS**

**Thesis  
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A brief report of part of the work described in  
Chapter Four has been published (Kelly, 1966).

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## SYNOPSIS

Evidence has accumulated which suggests that other things being equal the quantum content of the transmitter liberated from nerve terminals by the nerve impulse depends on (a) the concentration of calcium in the vicinity of the nerve endings (del Castillo and Katz, 1954 a,b; Katz and Miledi, 1965 c) and (b) the amplitude of the presynaptic action potential (Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966; Hubbard and Willis, 1962; Liley, 1956 c). However, one procedure which would be expected to depress the amplitude of the presynaptic action potential, namely a reduction in the extracellular sodium concentration, has been found to produce little effect on the output of acetylcholine in the presence of the normal calcium concentration. The purpose of the work described in this thesis is to explore the possible reasons for the apparent anomaly. The main experiments consisted of determining the way in which the quantal content of the end-plate potential was altered by variations in the ionic composition of the extracellular fluid.

Chapter 1 gives a brief review of the relevant literature, Chapters 2 and 3 describe the experimental methods and the way in which the quantal contents were calculated. Chapter 4 describes experiments in which extracellular sodium chloride was replaced by sucrose or

glycine. The results of these experiments show that in solutions containing low concentrations of calcium the quantal content of the end-plate potential was increased by the reduction in the concentration of sodium chloride and furthermore that the quantal content in solutions containing low amounts of sodium chloride was relatively insensitive to changes in the calcium concentration. An attempt was made to interpret these results on the basis that sodium and calcium normally compete for a membrane site which allows the liberation of acetylcholine only when occupied by calcium. The results, however, were not unequivocally consistent with this hypothesis. For this reason an alternative approach was adopted. It has already been established that magnesium reduces the output of acetylcholine by competition with calcium (Jenkinson, 1957). If therefore sodium competes with calcium for the same site as does magnesium, an interaction would be expected between the effects of sodium, calcium and magnesium on the output of acetylcholine. Such an interaction was looked for in the work described in Chapter 5 but it was not found. An alternative explanation for the observations described in Chapter 4 is that the increase in output of acetylcholine resulted from the reduced ionic strength of the bathing solution. If this were so, replacement of sodium chloride by substitutes which maintain the ionic strength but reduce the amplitude of the presynaptic action potential would

be expected to reduce the quantal content of the end-plate potential. The four experiments described in Chapter 6 show that replacement of sodium chloride by tris-(hydroxymethyl)-aminomethane, methylammonium and ethylammonium chlorides do indeed lead to a reduction in the quantal content. However, interpretation of these experiments is ambiguous; as is described in Chapter 7 the replacement of sodium chloride by lithium chloride has also been found to cause some reduction in the quantal content and this reduction may not be entirely attributable to a reduction in the amplitude of the presynaptic action potential.

THE RELEASE OF TRANSMITTER AT

NEUROMUSCULAR JUNCTIONS

## INTRODUCTION

The standard concept of cholinergic transmission is that at certain synapses the action potential in the presynaptic nerve fibre causes the release of acetylcholine from the nerve terminal; the acetylcholine then diffuses across the synaptic gap to react with specific post-synaptic receptors.

Although a great deal is known about the action of acetylcholine on the post-synaptic membrane and the transition of the local depolarization to a propagated action potential, little is known about the process whereby the acetylcholine is liberated from the nerve terminal by the presynaptic action potential. Two kinds of synaptic activity can be recorded postsynaptically with microelectrodes: (i) the depolarization evoked as a result of the nerve impulse (the end-plate potential, e.p.p., at the motor end-plate or synaptic potential in ganglion cells) which normally lead to action potentials, and (ii) a spontaneous form of activity, consisting of sub-threshold, miniature e.p.p.'s at the muscle end-plate or spontaneous synaptic potentials in ganglion cells; these occur at random intervals in the absence of any stimulation. (Fatt and Katz, 1952a,b; Nishi and Koketsu, 1960). There is good evidence reviewed by del Castillo and Katz (1954 b) and Katz (1958, 1962) that the spontaneous discharge results from



an intermittent release of multimolecular packets of acetylcholine from the nerve terminals.

The presynaptic action potential. In recent experiments, Katz and Miledi (1965 a) have taken advantage of the diffuse nature of the frog neuromuscular junction to elaborate on the earlier findings of Hubbard and Schmidt (1963). They suggested that active propagation of the action potential occurred into the motor nerve endings of the rat diaphragm. By recording through a single electrode both the presynaptic and the post-synaptic currents from localised synaptic spots at different distances from the origin of the synaptic tree, Katz and Miledi have shown that the nerve impulse, after reaching the end of the myelinated portion of the axon continues to propagate along the synaptic non-myelinated terminals.

Quantal nature of the release. At low temperatures Katz and Miledi (1965 e) found that the post-synaptic current which was recorded from only a few per cent of the total area of the end-plate, was made up of quantal components which behaved in an independent manner. The number of quanta varied from impulse to impulse in a random fashion as predicted by Poisson's theorem. The small number of quanta released at each active part of the terminal corresponded to the much larger value known to be appropriate for the whole junction.



Previously the number of quanta released from the entire nerve terminal by nerve impulses had been shown to fluctuate when the amount of acetylcholine released was depressed by a reduction in the extracellular concentration of calcium or an increase in the concentration of magnesium. Statistical analysis of these fluctuations had shown that the e.p.p.'s were composed of discrete units whose mean amplitude and variability were identical with those of the spontaneously, randomly occurring miniature e.p.p.'s (del Castillo and Katz, 1954 b; Martin, 1955; Boyd and Martin, 1956; Liley 1956 b). At other cholinergic synapses besides the neuromuscular junctions of the frog and mammal the post-synaptic potentials are also composed of varying numbers of quanta (for example, sympathetic ganglia of the frog - Blackman, Ginsborg and Ray, 1963; parasympathetic ciliary ganglia of the chick - Martin and Pilar, 1964). Estimates of the number of quanta normally released by a nerve impulse at the neuromuscular junction usually lie in the range between 100-300 for both amphibia (del Castillo and Katz, 1954b; Martin, 1955) and mammals (Boyd and Martin, 1956; Liley, 1956b; Takeuchi and Takeuchi, 1960a; Brooks and Thies, 1962).

The relationship of the presynaptic action potential to the release of transmitter. Depolarization of the presynaptic nerve terminals either by depolarizing currents (del Castillo and Katz, 1954d; Liley, 1956c) or by an increase in the extracellular potassium concentration (Liley, 1956c; Furukawa, Furukawa and Takagi, 1957; Birks, Huxley and Katz, 1960; Takeuchi and Takeuchi, 1961) leads to an increase in the frequency of the miniature e.p.p.'s. It was therefore suggested by Liley (1956c) that depolarization increased the probability of the spontaneous release of quanta and that the presynaptic action potential caused the release of acetylcholine simply by virtue of the depolarization it caused of the nerve terminal. An e-fold increase in the external potassium concentration which perhaps corresponds to a 26mV depolarization of the nerve terminals, was shown by Liley to cause the discharge rate of the miniature e.p.p.'s to rise by a factor of approximately  $e^4$ . He concluded that the rate of release of acetylcholine increases exponentially by a factor of 100 for a 30mV depolarization, and that a simple extrapolation of this relationship would account for the release of the large number of quanta during the depolarization imposed during even the brief period occupied by the presynaptic nerve impulse.

If Liley's hypothesis is correct, quite small changes in the size of the presynaptic action potential will be

reflected by large changes in the amount of transmitter released; for instance a 10mV reduction in the amplitude of the presynaptic action potential would cause about a 3.5 fold reduction in the amount of transmitter released. (See appendix to Chapter 1, p. 17). Of course the question arises, as pointed out by Katz (1962) whether the exponential relationship holds over the range of depolarizations that Liley was unable to examine. In frog muscle, for example, the relationship between the logarithm of the tension developed by a single twitch fibre in response to depolarization is linear over only part of the range and develops a maximum at about 60mV although the action potential reaches an amplitude of 120mV (Hodgkin and Horowicz, 1960).

The effect of depolarizing the presynaptic nerve terminals by the passage of current may not be identical with the effect of an increase in the extracellular potassium concentration. An increase in the potassium concentration causes an increase in quantal content of the e.p.p. (Liley, 1956d; Takeuchi and Takeuchi, 1961; Edwards and Ikeda, 1962; Hubbard and Willis, 1962; Parsons, Hofman and Feigen, 1965); however, e.p.p.'s recorded from the rat diaphragm are reduced in amplitude when the nerve terminal is depolarized electrically (Hubbard and Willis, 1962). Since the amplitudes of the miniature e.p.p.'s are not reduced, it appears that presynaptic depolarization reduces the quantal content.

Gage and Quastel (1965a) have concluded that in the rat-diaphragm the increase in the frequency of the miniature e.p.p.'s caused by an increase in the potassium concentration probably continues to develop after depolarization of the nerve terminals has reached a maximum. They have therefore, suggested that potassium ions may influence transmitter release by mechanisms distinct from that of depolarization of the nerve terminals.

Parsons, Hofman and Feigen (1965) have found that an increase in external potassium concentration not only increases the amount of transmitter released by a nerve impulse in the initial stages of a tetanus, but also raises the level at which the output can be sustained during the tetanus. They suggested that potassium raises the number of quanta of 'available' acetylcholine.

If part of the acceleration of the miniature e.p.p. frequency in excess potassium concentrations is due to an increase in the 'available' acetylcholine rather than exclusively to the degree of nerve terminal depolarization, as Gage and Quastel (1965a) have pointed out, the relationship between the rate of transmitter release and the degree of depolarization during the action potential would be less steep than that predicted by Liley (1956c). Depolarization of the terminals by potassium might lead to an increase in the intracellular sodium concentration

and Birks (1963) has suggested that it is this which is responsible for the increase in the 'available' acetylcholine. This idea is supported by the fact that interference with the 'sodium pump' by cardiac glycosides or by potassium deficiency leads to a progressive increase in the miniature e.p.p. frequency and an increase in the quantal content of the e.p.p. However, it must be noted that procedures which lead to an increase in sodium concentration will presumably cause a depolarization of the nerve terminal.

The relationship between the amount of transmitter released and the height of the presynaptic action potential has been examined quantitatively at the squid giant synapse where it is possible to record simultaneously from the pre- and post-synaptic nerves. (Hagiwara and Tasaki, 1958; Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966) The results of Takeuchi and Takeuchi (1962) can be plotted (see Eccles, 1964) to show that there is an approximately linear relationship between the logarithm of the amplitude of the excitatory post-synaptic potential and the amplitude of the presynaptic action potential. A similar relationship has been shown to exist between the logarithm of the amplitude of the e.p.p. and the amplitude of the presynaptic spike recorded externally at the rat neuromuscular junction by Hubbard and Schmidt (1963).

One feature of Liley's hypothesis is that the



relationship between depolarization and increased frequency of quantal release was assumed to be instantaneous. According to this the release of the majority of the quantal units should occur within the brief time interval occupied by the crest of the spike. However, it has recently been shown by Katz and Miledi (1965b, d, e) that the release of transmitter does not occur at the instant the action potential wave arrives at the junction but starts only after a delay of about 0.5 msec (at 20°C) and continues for a few milliseconds thereafter. Katz and Miledi (1965d) have also shown that when a brief negative going pulse is applied through a micropipette to the surface of a presynaptic nerve which had been treated with tetrodotoxin to prevent an action potential being initiated, the release of transmitter occurs after the end of the pulse. The amount released increased as the size of the current was increased. The delay between the pulse and the release fluctuated presumably as a consequence of the statistical nature of the quantal mechanism of transmitter release.

This brings the process of transmitter release into line with the other changes in the axon which result from a displacement of its membrane potential. The increase in ion permeability, first to sodium, then to potassium do not occur instantaneously when the membrane is depolarized but follow with a measurable time lag. The release of acetylcholine may well be the end product

of a sequence of reaction steps set off by the displacement of the membrane potential.

Calcium. The importance of calcium in synaptic transmission has been recognised since the work of Locke (1894). It is known that the release of acetylcholine by the nerve impulse fails if calcium is withdrawn from the external medium. In both amphibia and mammals withdrawal of calcium has been shown to reduce the size of the e.p.p. at the neuromuscular junction (del Castillo and Stark, 1952; Fatt and Katz, 1952a; del Castillo and Katz, 1954a,b; Liley, 1956b; Boyd and Martin, 1956) and the reduction in the output of acetylcholine has been confirmed by the more direct method of assaying the acetylcholine output from perfused sympathetic ganglia (Harvey and MacIntosh, 1940; Hutter and Kostial, 1954).

An increase in the calcium concentration increases the amount of transmitter released by the nerve impulse without causing any alteration in the amplitude of the focal nerve spike recorded extracellularly at the frog neuromuscular junction (Katz and Miledi, 1965c), the mammalian neuromuscular junction (Hubbard and Schmidt, 1963) or intracellularly from the presynaptic nerve of the giant synapse of *Loligo* (Takeuchi and Takeuchi, 1962; Miledi and Slater, 1966).

The importance of calcium ions in the process of transmitter release is not an isolated instance; it is



to some extent analogous to the role which calcium ions appear to play in the activation of muscular contraction (Niedergerke 1956a,b; Lüttgau and Niedergerke, 1958; Frank, 1960 and Durbin and Jenkinson, 1961). The presence of calcium ions or some calcium compound within the membrane seems to be required for a membrane depolarization to produce its effect, whether the specific result is the release of transmitter or the activation of contraction.

While calcium is an essential "co-factor" in the process of neural secretion, magnesium acts as a competitive antagonist to calcium.

Del Castillo and Katz (1954a,b) put forward the hypothesis that calcium and magnesium compete for a carrier molecule, X, in the nerve endings. On the arrival of a nerve impulse, the calcium compound alone breaks down to give calcium ions and an active form of X, X', which can release or allow the passage of acetylcholine. Thus the output of acetylcholine is dependent on the proportion of X which is combined with calcium.

Jenkinson (1957) has shown that there is a reasonable agreement between the experimental and theoretical relationship between the calcium and magnesium concentrations required to maintain the e.p.p. amplitude at a constant level on the hypothesis that the two ions compete for the same site.

Calcium on the spontaneous release of acetylcholine.

According to Liley's hypothesis, the quantal content of the evoked e.p.p. is a function of the resting frequency of spontaneous e.p.p.'s; since calcium lack reduces the quantal content of the e.p.p. it should also diminish the frequency of spontaneous quantal release at rest. However, Fatt and Katz (1952b) found that there was no consistent change in the miniature e.p.p. frequency when the calcium concentration was altered.

On the other hand in the mammalian preparation Liley (1956c) and Hubbard (1961) found that the change in frequency of the miniature e.p.p.'s paralleled the alteration in the amplitude of the e.p.p. which occurred when the calcium or magnesium concentration was altered. The miniature e.p.p. frequency bore a linear relationship to the logarithm of the calcium concentration over the range 0.5 to 10mM.

Two types of miniature e.p.p.'s. The indifference of the resting frequency of the miniature e.p.p.'s in the frog to either the calcium or the magnesium concentration is explained by Hubbard (1961) by postulating that there are two types of miniature e.p.p.'s, one being calcium independent and unrelated to the production of the e.p.p. and the other calcium dependent and constituting the component units of the e.p.p.'s. The postulate of a calcium independent

fraction of the miniature e.p.p.'s is based on the observation that miniature e.p.p.'s continue indefinitely in the absence of calcium ions or in the presence of a chelating agent and a high magnesium concentration, and the calcium dependent on the finding that calcium is essential for the potassium stimulated acceleration of the miniature e.p.p. frequency. The frequency of the calcium independent fraction is not affected by presynaptic depolarization by potassium ions and therefore is assumed not to contribute to the e.p.p.; (in contrast to the calcium dependent fraction their frequency is raised by the stress of stretching or of increasing the osmotic pressure). It appears that in the frog under resting conditions almost all the miniature e.p.p.'s are of the calcium independent type, hence there arises the discrepancies between the miniature e.p.p. frequency and the size of the e.p.p.

In the mammalian preparation, the resting frequency of the miniature e.p.p.'s may be regarded as a measure of the probability of release of a quantum; the increased resting frequency in raised calcium concentrations may be interpreted as an increase in the probability of release of the individual quantum by increased extracellular calcium. In the frog, however, it appears that the probability of release of a quantum is reflected not by the resting frequency but by the intensity of the potassium induced acceleration of the miniature e.p.p.

frequency. This measure should also, of course, apply to the mammalian preparation.

Sodium. An early hypothesis was that the ACh. was discharged from the nerve terminal in exchange for the sodium ions which entered during its electrical activity (Fatt and Katz, 1952c). However, the reduction in the amplitude of the e.p.p. which they observed when part of the extracellular sodium was replaced by sucrose was later shown to be largely due to a decrease in the post-synaptic sensitivity to ACh. (Nastuk 1953; del Castillo and Katz 1955b). Even stronger evidence against the idea that there was a specific exchange between sodium and acetylcholine ions came from the observation by del Castillo and Katz (1955b) that the spontaneous quantal release continues when all the sodium chloride was replaced by potassium sulphate. Other studies have shown that the potassium induced release of ACh. can occur from ganglia which have become inexcitable by perfusion with sodium free sucrose Ringer (Hutter and Kostial, 1955; Birks, 1963).

Direct estimates of the ACh. output of stimulated preganglionic nerve endings by Hutter and Kostial (1955) revealed no reduction in the release until the sodium concentration of the perfusion fluid was reduced to a level likely to produce nerve block. However, at higher stimulation rates, 20/sec, a reduction in the output of



acetylcholine has been reported to occur in the presence of low sodium concentrations (Birks, 1965). In the slow fibres of the frog, Burke (1956) found that the amplitude of the synaptic potentials were little reduced when the sodium chloride in the extracellular fluid was exchanged for sucrose. There is little evidence that a reduction in extracellular sodium causes a reduction in the amount of transmitter released in response to nerve stimulation in the presence of the normal calcium concentration. The effect of changes in the external sodium concentration is considered further in subsequent chapters of this thesis.

#### The effects of tubocurarine and neostigmine.

Many of the experiments described in the subsequent chapters were carried out in the presence of sufficient tubocurarine to depress the amplitude of the e.p.p. to less than 5mV. Recently the question has arisen as to whether the action of tubocurarine can be explained solely by a post-synaptic antagonism of the depolarizing action of acetylcholine on the junctional membrane (Riker, 1960; Standaert, 1964).

In 1961, Lilleheil and Naess revived the theory that the gradual decline in size of successive e.p.p.'s during the initial period of a tetanus is caused by a decrease in the amount of transmitter released by successive stimuli due to the presynaptic action of tubocurarine. Since tubocurarine had been shown to have no influence on

the actual amount of acetylcholine released from the perfused muscle during stimulation at 15/sec (Dale et al, 1936; Emmelin and MacIntosh, 1956), Lilleheill and Naess assumed that tubocurarine has no presynaptic action unless the rate of nerve stimulation is in excess of 15/sec. However, the neuromuscular depression they attributed to tubocurarine can be observed at stimulation rates as low as 0.25/sec (Takeuchi, A., 1958) and can occur in the complete absence of tubocurarine (Blackman, 1963). In addition Otsuka, Endo and Nonomura (1962) showed that the rate of decline of the amplitude of the e.p.p. during a tetanus was independent of the concentration of tubocurarine and therefore, unrelated to the presence of tubocurarine. More recently Beani, Bianchi and Ledda (1964) have reported that the release of assayable acetylcholine during a high frequency volley is depressed in the presence of tubocurarine. However, they were unable to demonstrate that the release of transmitter was depressed by tubocurarine when the frequency of stimulation was reduced to 6/sec.

During the present series of experiments the frequency of nerve stimulation was 0.31/sec which is similar to that used by Martin (1955) at the neuromuscular junction who showed that the quantal content of the e.p.p. is unaltered by the presence of tubocurarine.

Neostigmine. Recently it has been suggested that anticholinesterases prolong transmitter activity by a presynaptic action (see Werner and Kuperman, 1963) rather than by the inhibition of cholinesterase (Eccles, Katz and Kuffler, 1942; Eccles and MacFarlane, 1949; Fatt and Katz, 1951). Prostigmine was only occasionally used to enhance the amplitude of the spontaneous and evoked e.p.p.'s because of the striking change in the shape of the e.p.p. which occurs in the presence of an anticholinesterase when the neuromuscular junction is blocked by the reduction of the external sodium to one-fifth (Fatt and Katz, 1951). Instead of rising quickly to a sharp peak, the e.p.p. rises to a plateau which is maintained for some 30 - 40 msec and then decays to one-half in 0.1 sec, as compared with 6 msec in the absence of an anticholinesterase. Since no similar change in shape has been reported for either the miniature e.p.p.'s or the artificial e.p.p. produced by the iontophoretic application of acetylcholine to the end-plate (Fatt and Katz, 1952c; del Castillo and Katz, 1955) it might be that anticholinesterases modify the release of transmitter when part of the sodium chloride in Ringer is replaced by sucrose.



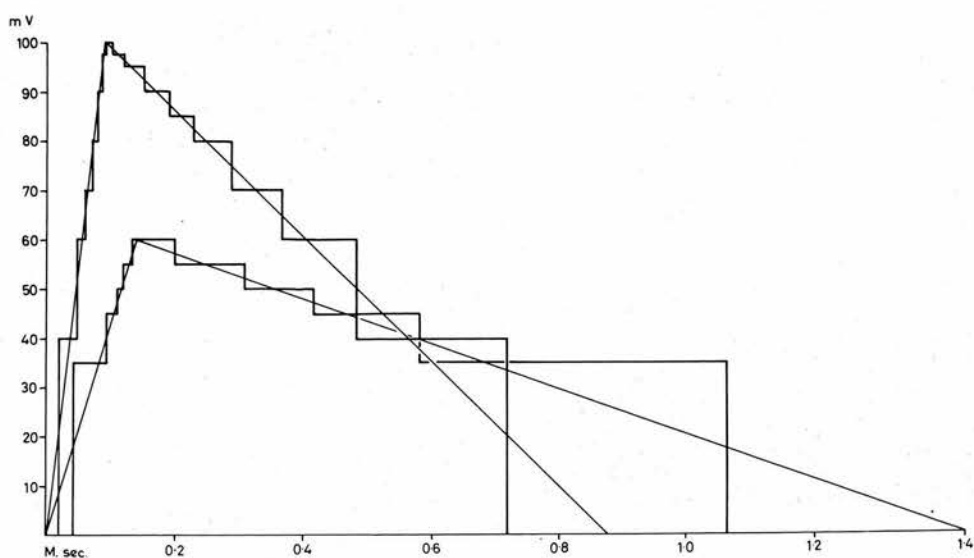
## APPENDIX

The relationship between the quantal content and the amplitude of the presynaptic action potential.

Liley (1956c) predicted that during the depolarization of the nerve terminal caused by the presynaptic action potential, the resting miniature e.p.p. discharge frequency increases by a factor of 100 for every 30mV the nerve terminal is depolarized. On this assumption, the number of quanta that would be expected to be released by an action potential of any particular amplitude or duration can be derived as shown in Fig. 8 of Liley's paper. Fig. (1.1) shows two action potentials, one of 100 mV which has the same amplitude and duration as that described by Liley for a mammalian motor nerve and a second drawn with an amplitude of 60 mV. For the sake of simplicity, it was assumed that the shape of an action potential approximates to a triangle and that when the amplitude is reduced, there is a proportional increase in the duration which maintains the area of the triangle. Both action potentials have been divided into a series of steps of known duration and depolarization and from the expected increase in the miniature e.p.p. frequency with depolarization during each step the number of quanta released by each action potential calculated. On the assumption that the resting miniature e.p.p. discharge frequency was 1/sec a reduction in the amplitude of an action potential from 100 to 60 mV would

be expected to reduce the quantal content by a factor of 115 from 239 quanta to 2.

Table (1.2) compares the number of quanta that would be expected to be released by each depolarization step during the passage of 5 action potentials of different amplitudes. The amplitudes of the action potentials were selected on the basis that a 100 mV action potential would be expected to be reduced to 83, 73 and 60 mV when the sodium chloride concentration is reduced to one-half, one-third and one-fifth of the normal respectively (the amplitude falls 58 mV for every 10 fold reduction in the external sodium concentration (Hodgkin and Katz, 1949; Huxley and Stampfli, 1951)). The table shows that a reduction in the amplitude of the action potential from 100 to 90, 83, 73 and 60 mV would be expected to reduce the quantal content by a factor of 3.5, 6.5, 29 and 115 respectively.



**Fig. (1.1)** Two action potentials of 100 and 60 mV divided into a number of steps of known duration and level of depolarization. From the relationship between the level of depolarization predicted by Liley (1956c) it was calculated that the 100 mV action potential would release 115 times more transmitter than the smaller.

Table (1.1) The number of quanta that would be expected to be released by each depolarization step of five action potentials with amplitudes between 100 and 60 mV. Not all of the action potentials were divided into steps of the same level of depolarization and hence there are gaps in the table.

Depolarization during each step	No. of Quanta released per msec	Amplitude of Action Potential (No. of quanta released in each step)				
		100	90	83	73	60
100	3981	39.8				
97.5	2661	59.4				
95.0	1778	56.7				
90.0	1000	46.0	28.0			
85.0	398	18.3	19.1			
83.0	316			5.7		
80.0	178	13.1	14.3	19.9		
75.0	89			5.6		
73.0	63.1				1.5	
70.0	42.6	3.9	4.3	4.1	4.4	
60.0	10.0	1.3	1.1	1.2	1.68	0.68
55.0	4.5					0.85
50.0	2.0		0.3	0.4	0.50	0.24
45.0	1.0					0.19
40.0	0.5	0.2				
35.0	0.2					0.12
30.0	0.1		0.03	0.03	0.05	
Total number of quanta		238.6	67.13	36.93	8.13	2.08

## **CHAPTER 2**

### **METHODS**



### A. Preparations.

Either the sartorius or the extensor digiti IV muscle of the frog (*Rana pipiens*) was employed depending on whether e.p.p.'s were to be recorded intracellularly from a single muscle fibre or externally from the whole muscle. The experiments were carried out throughout the year on frogs stored at 4°C for up to 3 months.

### B. Solutions.

'Normal' Ringer solution had the following composition (mM): sodium 115.6; potassium 2.0; calcium 1.8; chloride 119.4. Usually, the addition of about 2 ml per litre of isotonic phosphate buffer solution containing 63 mM  $\text{Na}_2\text{HPO}_4$  and 23 mM of  $\text{NaH}_2\text{PO}_4$  per litre was necessary to maintain the pH between 6.5 and 6.8.

Sodium deficient solutions were prepared by mixing normal Ringer with isotonic solutions, of the sodium substitute under study, which contained the same concentration of potassium and calcium as the normal Ringer and sufficient phosphate buffer to maintain the pH.

### C. Recording Equipment.

Conventional recording arrangements were used (Fatt and Katz, 1951). The first stage consisted of a double sided cathode follower using ME-1400 valves. The input valve was checked for a low grid current in normal operating conditions by measuring the voltage drop across a high resistance (500 M $\Omega$ ) connected between the grid

and earth. The selected valve had a grid current of  $4 \times 10^{-12}$  A.

The chlorided silver wire connecting the micro-electrode to the input grid was kept short, and the micro-electrode shielded, the shield being connected to the cathode to reduce the input capacitance of the system (Nastuk and Hodgkin, 1950). The bath electrode consisted of a chlorided silver wire embedded in agar-Ringer and was connected through a calibrator to the earthed side of the cathode follower. The output of the cathode follower was led through a short shielded cable and preamplifier (100x) to the inputs of one of the differential amplifiers of a Tektronix oscilloscope (model 502).

Time constant. The input capacitance of the recording system was determined by applying a square pulse between earth and a known screened resistor of  $10 \text{ M}\Omega$  connected in series with the input grid. Since the capacitance of the microelectrode to earth depends upon the length of the tapered portion dipping into the bath this was carefully adjusted to that used in the actual experiments. The input capacitance C was calculated from the expression:-

$$\tau = \frac{RR_1}{R_1 + R} \cdot C$$

where  $\tau$  is the time in  $\mu\text{sec}$  taken for the output voltage which was observed on the oscilloscope to rise exponentially to 63% of the final steady voltage, R is the



known resistance of  $10\text{ M}\Omega$ , and  $R_1$  is the resistance of the microelectrode in  $\text{M}\Omega$ . The input capacitance of the system with a microelectrode dipping 2mm below the surface of the Ringer fluid was  $4.4\text{ pF}$  i.e. with a  $10\text{ M}\Omega$  resistance electrode the time constant of the system was  $44\text{ }\mu\text{sec}$ .

Noise. When miniature e.p.p.'s were recorded with this system at slow sweep speeds and at high gain they were difficult to measure because of the base-line noise. In order to increase the signal to noise ratio the band width of the system was reduced to  $2.75\text{ Kc/s}$  by introducing a low pass filter with a time constant of  $57.6\text{ }\mu\text{sec}$  between the preamplifier and the oscilloscope. The output of the preamplifier was connected to the input terminals of the oscilloscope through two  $18\text{ K}\Omega$  resistors and the input terminals of the oscilloscope were shorted by a  $1600\text{ pF}$  capacitor. The filter satisfactorily reduced the amplitude of the base line noise without any attenuation of the e.p.p.'s or m.e.p.p.'s. When the same e.p.p.'s recorded with and without the filter, were displayed simultaneously on the two channels of the oscilloscope, their shapes and amplitudes were identical.

#### D. Intracellular recording.

The sartorius muscle and its nerve were dissected and mounted with its deep surface up in a small 'Perspex' chamber. The muscle was stretched to about 125% of its resting length and secured by silk threads tied to the

pelvic and tibial tendons.

Stimulation. A short length of the nerve was sucked into a capillary electrode similar to that described by Furshpan and Potter (1959). A platinum wire dipping into the fluid inside the capillary formed the anode and a platinum loop around the tip - the cathode. Stimuli were applied to the nerve from a square wave generator through an isolation transformer.

Perfusion System. Several systems were employed to change the solutions in the muscle chamber as rapidly and completely as possible without dislodging the recording electrode.

At first, the preparation was mounted in a large circular bath containing about 40 ml of solution and the bath fluid was changed by the addition of about 200 ml of solution from a plastic wash bottle while the fluid level in the bath was maintained constant during the exchange, by means of suction applied to a capillary tube connected to a water pump through a siliconed trap; it was necessary to use at least four times the bath volume to effect the total replacement of the original bath content.

Most of the experiments, however, were performed with a recording chamber 1.5 cm wide and 10 cm long; the depth varied between 0.6 cm at the ends to 0.2 - 0.3 cm

in the middle. The bath contained approximately 5 ml of solution. Solutions entered the chamber at one end and were drawn off by suction through a bevelled hypodermic needle from the surface at the other. Both the inlet and the outlet were screened from the muscle by baffles made of nylon netting (taken from the filter chamber of disposable 'blood giving sets') which smoothed the flow over the muscle and prevented dislodgement of the recording electrode by bubbles. The tissue was positioned so that the main end-plate region was near the centre shallow part of the bath.

Solutions were changed either by squirting fluid vigorously from a wash bottle into the inlet 'well' formed between the end of the bath and the net baffle or if more than a single change of solution was required by a more sophisticated system. Solutions were placed in 100 ml separating funnels about 75 cm above the level of the muscle, whence they flowed by gravity to a multiple-tap stopcock (Hodgkin and Horowicz, 1959) immediately adjacent to the recording chamber. The volume of solution in the perfusion line between the stopcock and the bath was kept to a minimum. Flow rates of about 2 ml/sec could be achieved and although in practice 50 ml of solution or more were used, 25 ml were sufficient to flush methylene blue from the chamber.

The recording electrodes were inserted by conventional means into the end-plate region which was located visually with the aid of a binocular dissecting microscope. The location of an electrode was considered to be satisfactory only if the 'rise time' of the miniature e.p.p.'s or, in curarized preparations of the e.p.p.'s, was less than 2 msec.

Microelectrodes. The microelectrodes were pulled by a machine similar to that described by Frank and Fuortes (1955) from pyrex glass (2 mm o.d. and 1 mm i.d.) and filled with 3 M KCl by boiling under reduced pressure. Electrodes with resistances of 7 to 14 M $\Omega$  were used. The resistances were measured by applying a steady current to the electrode and measuring the potential difference across the electrode  $V_1$  with a Vibron Electrometer Model 33 B-2. The input terminals were then shunted with a 10 M $\Omega$  resistance and a second voltage  $V_2$  was observed. The microelectrode resistance was then equal to:-

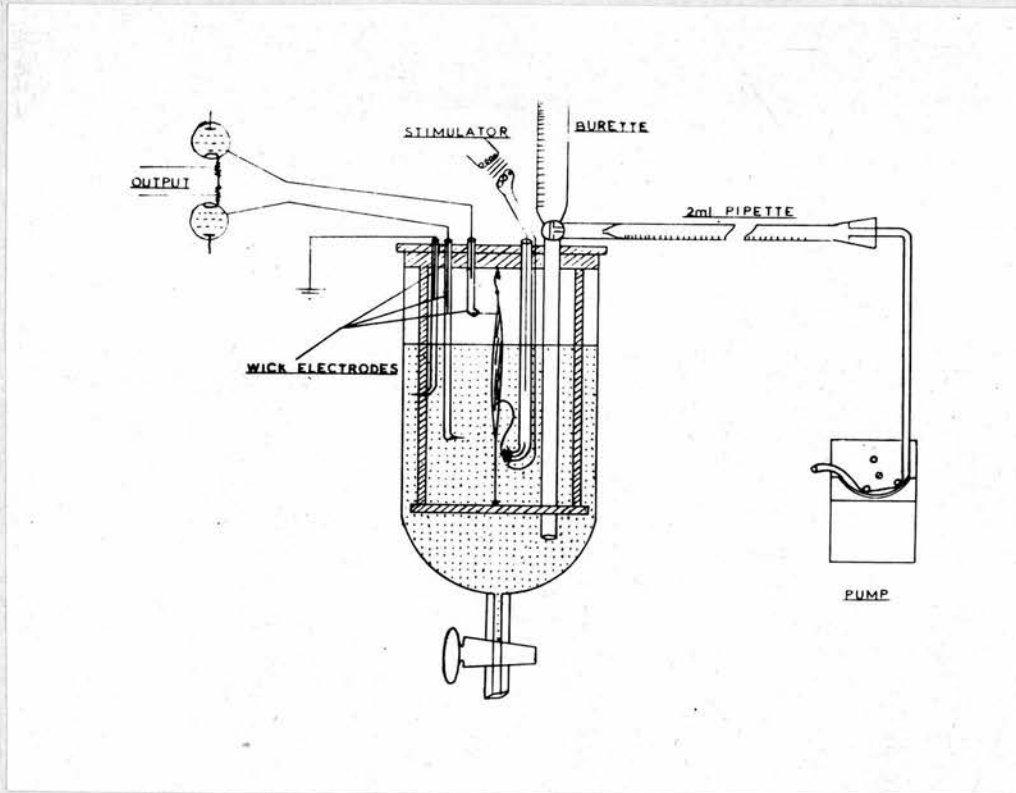
$$10 \times \frac{V_1 - V_2}{V_2} \text{ M } \Omega$$

In very much the same way the electrode resistance could also be determined during an experiment by applying a square pulse between the bath electrode and earth and observing the output  $V_1$  before and  $V_2$  after the microelectrode was shunted to earth by a 10 M $\Omega$  resistance.

### E. Extracellular recording.

The extensor digiti IV, was suspended vertically in a cylindrical bath (volume 130 ml) made of glass provided with three silver-silver chloride electrodes with thread wicks (see Fig.211). Muscles with slips to the 3rd toe were discarded. The muscle was mounted upside down between a stainless steel loop in the centre of the stopper and the middle of a 'Perspex' stirrup mounted 7.5 cm below, by means of a siliconed glass hook tied to the distal or uppermost tendon and a silk ligature tied between the proximal or lower tendon and the centre of the stirrup. The length of the thread was adjusted until the muscle was under sufficient tension to regain its in vivo resting length. The muscle was mounted upside down because the distal end is more suitable for locating a wick electrode since it is free of nerves and end-plate regions. The nerve which can be left attached to the proximal tendon, adheres to the silk ligature and gains support when the bath is drained. The nerve was sucked into a capillary stimulating electrode similar to that described earlier, which was also designed to support the nerve when the bath was drained. One of the two recording electrodes was placed on the uppermost tendon just below the glass hook and the other immersed in the solution below the level of the end-plate region of the muscle. With this arrangement the air-Ringer interface acted as a moveable electrode (Fatt, 1950) and was





**Fig. (2.1)** Bath used for extracellular recording of e.p.p.'s for the end-plate region of the extensor digiti IV muscle - see text for detailed description.

used to locate the maximum density of end-plates i.e. the position where the e.p.p. with the greatest amplitude and fastest rise time could be recorded. The third electrode was also immersed in the solution and connected to earth and the responses were recorded differentially to avoid mains interference and to reduce the size of the stimulus artifact.

During the 'rest' periods, the bath was filled with solution to the base of the glass hook and the procedure for locating the end-plate region began by complete drainage of the bath through the stopcock in the base. The nerve was stimulated supramaximally every 2 sec and the bath was slowly filled through a glass tube reaching to below the stirrup connected to a 100 ml burette and the volume (usually 82-88 ml) noted at which the amplitude and rise time of the e.p.p. had just passed its maximum. The maximum size of the e.p.p. was then measured from the continuous sequence of e.p.p.'s recorded on moving film at one every two seconds as 1 - 1.5 ml of fluid were withdrawn into a 2 ml pipette mounted horizontally from the burette delivery tube. The fluid was sucked into the pipette at a constant rate of 1.875 ml/minute by means of a Watson-Marlow Flow Inducer (Model MHRE). If the profile of the responses was convex, the end-plate region had been satisfactorily located (see Fig.22). The region was scanned four more times as the same volume of fluid was pumped to and fro

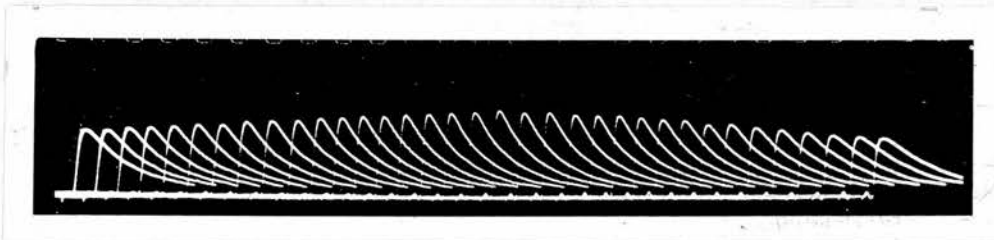


Fig. (2.2) Series of e.p.p.'s recorded extracellularly from the end-plate region of the extensor digiti IV muscle at 2 sec intervals on moving film as fluid was withdrawn from the bath at the rate of 1.875 ml/min. The convex profile of the responses indicates that the end-plate region has been satisfactorily located. The maximum amplitude of the e.p.p. was 2.1 mV.

between the pipette and the bath while the sequences of e.p.p.'s were superimposed on static film since only the maximum amplitude of the e.p.p. was of interest (see Fig.2.2). The maximum amplitude of the e.p.p. was taken to be the mean amplitude of the largest e.p.p.'s recorded during each of the five runs. Since the area of cross section of the bath was  $82.35 \text{ cm}^2$  the addition of 1 ml raised the level of the meniscus 0.012 cm and often 0.3 ml was sufficient to traverse the centre of the end-plate region.

The e.p.p.'s were usually recorded with the amplifier capacity coupled to avoid base line fluctuation and to aid the superimposition of e.p.p.'s. It was always ensured during each experiment that the amplitude of the e.p.p. was the same whether recorded A.C. or D.C.

### CHAPTER 3

A ESTIMATION OF THE NUMBER OF QUANTA  
RELEASED PER NERVE IMPULSE

B COMPARISON OF THE MEAN AMPLITUDE OF A  
SERIES OF E.P.P.'S RECORDED FROM  
CURARIZED MUSCLE BATHED IN  
FOUR DIFFERENT SOLUTIONS



A. Estimation of the number of quanta released per nerve impulse.

A large number of experiments consisted of recording with an intracellular electrode a sequence of (at least 100) evoked e.p.p.'s and as many spontaneous miniature e.p.p.'s as was convenient (50-100). The quantal content of the e.p.p.'s were then calculated as follows.

When the mean amplitude of the evoked response was less than 2 mV the amplitudes of successive responses could be analysed to give three estimates  $m_0$ ,  $m_1$ , and  $m_2$  of the number of quanta released per nerve impulse ( $m$ ). These estimates depend on the basic assumptions of the quantal hypothesis which were recently reviewed by Martin (1966).

When the amount of transmitter released by nerve impulses was sufficiently small a number of the impulses failed to release any quanta and were counted as 'failures'. The proportion of these failures ( $f$ ) to the number of stimuli ( $N$ ) gave an estimate  $m_0$  of  $m$ .

$$m_0 = \ln N/f \dots \dots \dots (1)$$

A second estimate of  $m$ ,  $m_1$  is given by the ratio of the mean amplitude of the evoked e.p.p.'s ( $\bar{v}$ ) to the mean amplitude of the spontaneous miniature e.p.p.'s

$$m_1 = \frac{\bar{v}}{\bar{v}_1} \dots \dots \dots (2)$$

A third estimate of  $m$ ,  $m_2$  could be made from the variance  $V = \left\{ \sum (v - \bar{v})^2 \right\} / N$  and the square of the mean amplitude of the end-plate potentials  $\bar{v}^2$ .

$$m_2 = \frac{\bar{v}^2}{V} \left( 1 + \frac{\delta^2}{\bar{v}^2} \right) \dots \dots \dots (3)$$

where  $\delta^2$  is the variance of the amplitude of individual quanta i.e. the variance of the amplitudes of the miniature e.p.p.'s. Often  $(1 + \delta^2 / \bar{v}^2)$  was ignored, as its experimental value was less than 1.05, and the estimate  $m_2$  was made without reference to the miniature e.p.p.'s.

$$\text{Then } m_2 \approx \frac{\bar{v}^2}{V} \dots \dots \dots (4)$$

Non-linear summation. When the amplitudes of the evoked e.p.p.'s were greater than 2 mV they were corrected for 'non-linear summation' of their component units. The correct amplitude  $v'$  was calculated from the observed amplitude of the e.p.p.'s by the formula given by Martin (1955).

$$v' = \frac{E}{E - v} \cdot v \dots \dots \dots (5)$$

where  $E$  is the difference between the resting membrane potential and the acetylcholine equilibrium or reversal potential. Therefore when the amplitude of the e.p.p. exceeds 2 mV (2), (3) and (4) become

$$m'_1 = \frac{\bar{v}'}{\bar{v}_1} \dots \dots \dots (6)$$

$$\text{and } m_2' = \frac{\bar{v}_1'}{v} \left(1 + \frac{\delta^2}{\bar{v}^2}\right) \dots \dots \dots (7)$$

$$m_2' \approx \frac{\bar{v}_1'^2}{v} \dots \dots \dots (8)$$

Recently Martin and Pilar (1964) and Martin (1966) have shown that the weighted mean  $m_3$  of  $m_1$  and  $m_2$  is a satisfactory estimate of  $m$ .

$$m_3 = \sqrt[3]{m_1^2 \cdot m_2} \dots \dots \dots (9)$$

The weighted mean  $m_3$  may be preferable to the other estimates of  $m$  as it avoids the tedious calculations involved in correcting the amplitude of each e.p.p. in a series. More important, however, was the fact that it provides a good estimate of the quantal content of the e.p.p. without requiring an accurate measurement of the resting potential or the acetylcholine equilibrium potential.

Equilibrium Potential. The acetylcholine equilibrium potential is usually assumed to be approximately -15 mV (Fatt and Katz, 1951; del Castillo and Katz, 1954c; Martin, 1955; Takeuchi and Takeuchi, 1960a). However the equilibrium potential is related to the extracellular concentration of sodium and potassium ions. When the extracellular sodium was replaced by sucrose the equilibrium potential was calculated from the equation below which was derived by Takeuchi and

Takeuchi (1959, 1960) to describe how the equilibrium potential for the end-plate current changed with the external concentration of sodium and potassium ions.

Equilibrium potential =

$$58 \left\{ \log_{10} \frac{[K]_o}{[K]_i} + \frac{\Delta_{gNa}}{\Delta_{gK}} \log_{10} \frac{[Na]_o}{[Na]_i} \right\} \cdot \left\{ 1 + \frac{\Delta_{gNa}}{\Delta_{gK}} \right\}^{-1}$$

where  $\Delta_{gNa}$  and  $\Delta_{gK}$  are the increases in sodium and potassium conductance at the end-plate membrane produced by acetylcholine and the other symbols have their usual significance. Takeuchi and Takeuchi (1960) have shown that  $\Delta_{gNa} / \Delta_{gK}$  remains fairly constant at 1.29 regardless of changes in the external sodium concentration;  $K_i$  and  $Na_i$  were taken to be 126 and 15.5 mM respectively.

B. Comparison of mean amplitudes of a series of e.p.p.'s recorded from curarized muscle bathed in four different solutions.

When the output of transmitter is high; the quantal content cannot be measured directly and changes in the output of transmitter must be inferred from the way in which the amplitudes of the e.p.p.'s in curarized muscle alter. In a number of experiments described in chapter 4 it was necessary to compare the amplitude of the e.p.p. of curarized muscle in four different ionic environments. The standardized procedure described below was adopted in an attempt to eliminate errors due to gradual changes which take place in the amplitude of the e.p.p. during the course of an experiment.

In each experiment e.p.p.'s were recorded by intracellular electrodes from end-plates of sartorii muscles bathed in solutions which contained sufficient tubocurarine to reduce the maximum amplitude of the e.p.p.'s to less than 5 mV. In each experiment four solutions A, B, C, and D were prepared by the dilution of a normal Ringer solution which contained a high calcium concentration, with a calcium free Ringer solution and a sucrose Ringer solution which contained neither sodium nor calcium. The solutions all contained the same tubocurarine concentration. Solutions A and B both contained the normal sodium concentration and B the normal and A twice the normal calcium concentration. C and D contained half of the normal



sodium concentration and the calcium concentrations were complimentary to those of solutions A and B respectively.

After a satisfactory end-plate was located a series of ten e.p.p.'s, at one every 10 sec, were recorded in each of the solutions A, B, C and D. The solutions were usually tested in the order A B A C D C A. Let the mean amplitude of the responses in each series be  $A_1$ ,  $B$ ,  $A_2$ ,  $C_1$ ,  $D$ ,  $C_2$ ,  $A_3$  then the following ratios were calculated

$$\frac{A_1 + A_2}{2B}, \quad \frac{(C_1 + C_2)(A_1 + A_2)}{2B(A_2 + A_3)}, \quad \frac{D(A_1 + A_2)}{B(A_2 + A_3)}$$

In effect the mean amplitudes of the e.p.p.'s in solutions A, C and D were expressed in terms of the mean amplitude of the e.p.p.'s recorded in solutions B.

## **CHAPTER 4**

### **THE EFFECT OF REPLACEMENT OF SODIUM CHLORIDE BY SUCROSE**

Partial replacement of sodium chloride in Ringer solution by sucrose reduces the amplitude of the nerve action potential, and its reduction is linearly related to the logarithm of the sodium concentration of the bathing fluid. (Hodgkin and Katz, 1949; Huxley and Stampfli, 1951; Ritchie and Straub, 1957). A reduction in the height of the presynaptic action potential would be expected to decrease the amount of transmitter released by the nerve impulse. However, as was mentioned in the introduction, Hutter and Kostial (1955) found that the amount of acetylcholine released from the superior cervical ganglion of the cat by nerve stimulation was unaltered by a reduction in the sodium concentration of the perfusion fluid. Also, del Castillo and Katz (1955) found that in non-curarized muscle the reduction in the amplitude of the evoked e.p.p. when the sodium concentration was reduced was no greater than the reduction which occurred in the amplitude of the miniature e.p.p. or of the depolarization caused by acetylcholine applied directly to the end-plate by iontophoresis. In slow fibres in frog muscle, Burke (1956) found that the amplitude of the junctional potentials was fairly insensitive to a reduction in the extracellular sodium concentration.

In the results referred to above, the calcium concentration in the bathing fluids was maintained at approximately the normal physiological level. In the

present chapter it will be shown that when the calcium concentration is reduced to one-eighth of the normal, a reduction in the sodium concentration causes an increase in the quantal content of the e.p.p.'s (cf. Birks and Cohen, 1965; Kelly, 1965). As has already been suggested a simple hypothesis (Birks and Cohen, 1965; Kelly, 1965; Gage and Quastel, 1965b) which would explain this interaction between sodium and calcium ions, is that these ions compete for a single receptor or strategic site and that the output of acetylcholine depends upon the number of sites occupied by calcium (cf. del Castillo and Katz, 1954a,b). Accordingly, two effects on the output of transmitter would result from a reduction in the extracellular sodium concentration. The presynaptic action potential would fall and lead to a reduction in the output of transmitter; however, an effect in the opposite direction would be obtained as a result of an increase in strategic sites occupied by calcium ions. At a particular calcium ion concentration these two effects might balance and if this concentration were close to the concentration in the solutions used by previous workers (Hutter and Kostial, 1955; del Castillo and Katz, 1955; and Burke, 1956), this would explain the apparent independence of the observed output on the sodium concentration. As will be shown below the hypothesis that sodium and calcium ions compete for a single site leads to the conclusion that at low

calcium concentrations a reduction in sodium should lead to a relative increase in the output of transmitter; at high calcium concentrations a relative fall should ensue.

## THEORY

### Scheme I

Suppose that calcium and sodium ions compete for some anionic group X on the cell surface to form either a calcium compound CaX which in some way participates in the release of acetylcholine, or an inactive compound Na<sub>2</sub>X:



By the law of mass action

$$\frac{[\text{Na}]^2 \cdot [\text{Ca X}]}{[\text{Na}_2 \text{X}] \cdot [\text{Ca}]} = K \dots\dots\dots(2)$$

However, if the total number of anionic groups is X<sub>0</sub> and these groups are occupied by either Ca or Na then:

$$X_0 = \text{Na}_2\text{X} + \text{Ca X} \dots\dots\dots(3)$$

$$\therefore \frac{[\text{Na}]^2 [\text{Ca X}]}{[X_0 - \text{Ca X}] [\text{Ca}]} = K \dots\dots\dots(4)$$

$$\therefore \frac{[\text{Ca X}]}{[X_0 - \text{Ca X}]} = \frac{K [\text{Ca}]}{[\text{Na}]^2} \dots\dots\dots(5)$$

The relationship between the output of acetylcholine and CaX is unknown but X<sub>0</sub> is a constant and if the output is assumed to be proportional to Ca X then the output



will be a function of  $\frac{[Ca]}{[Na]^2}$  i.e. if the amount of acetylcholine released is assumed to be equal when an equal number of receptor sites are occupied by calcium ions,

$$\frac{[Ca_1]}{[Na_1]^2} = \frac{[Ca_2]}{[Na_2]^2} \dots\dots\dots(6)$$

where the amount of transmitter released in the presence of  $[Na_1]$  and  $[Ca_1]$  is identical with the amount released in the presence of  $[Na_2]$  and  $[Ca_2]$ .

An identical relationship for the interaction of calcium and sodium on the tension developed by depolarized strips of cardiac muscle has been observed by Lüttgau and Neidergerke (1958).

## Scheme II

Another possibility is that the competition between sodium and calcium ions is not like an ion exchange mechanism and that the receptor sites exist in the free form as well as in the combined form with calcium or sodium ions.



Applying the laws of mass action

$$\frac{CaX}{X_0} = \frac{K_{Ca} [Ca_1]}{1 + K_{Ca} [Ca_1] + K_{Na} [Na_1]^2} \dots\dots\dots(9)$$

where  $X_0$  is the total number of receptors and  $K_{Ca}$  and

$K_{Na}$  are affinity constants.

Since it is assumed that when equal amounts of transmitter are released, equal numbers of receptor sites are occupied by calcium ions,

$$\frac{[Ca_1]}{1 + K_{Ca} [Ca_1] + K_{Na} [Na_1]^2} = \frac{[Ca_2]}{1 + K_{Ca} [Ca_2] + K_{Na} [Na_2]^2} \quad (10)$$

where the amounts of transmitter released in the presence of  $[Na_1]$  and  $[Ca_1]$  is identical with the amount released in the presence of  $[Na_2]$  and  $[Ca_2]$

From (10)

$$\frac{[Ca_1]}{1 + K_{Na} [Na_1]^2} = \frac{[Ca_2]}{1 + K_{Na} [Na_2]^2} \dots\dots\dots (11)$$

Equations (6) and (11) are most conveniently interpreted graphically. If the relationship between the output of acetylcholine and  $\log Ca$  is known for any particular sodium concentration then the relationship at another sodium concentration may be obtained by a parallel displacement of the original curve (see Fig. 4.1). Thus according to equation (6):

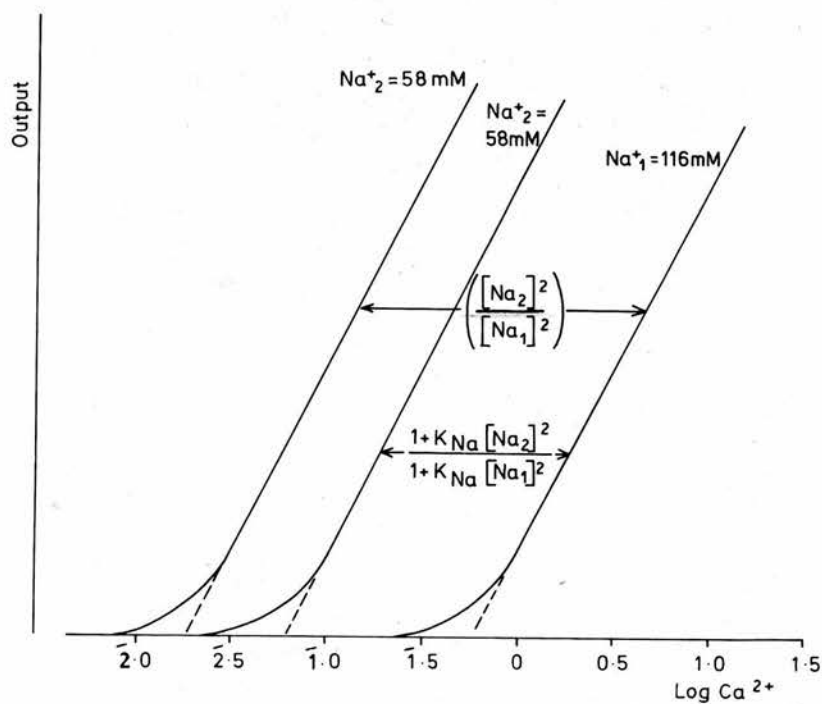
$$\log [Ca_2] - \log [Ca_1] = 2 \log \frac{[Na_2]}{[Na_1]} = \text{constant} \dots (12)$$

for a  
constant sodium  
concentration ratio

and according to equation (11)

$$\log [Ca_2] - \log [Ca_1] = \log \frac{1 + K_{Na} [Na_2]^2}{1 + K_{Na} [Na_1]^2} \dots\dots\dots (13)$$

Fig. (4.1)



**Fig. (4.1)** The output/log calcium curves in normal  $[\text{Na}_1^+] = 115.6$  and one-half  $\text{Na}^+$ ,  $[\text{Na}_2^+]$ , to show the displacements to the left that occur on the basis of equations (6) and (11).

The displacement predicted by equation (11) is evidently smaller than that predicted by equation (6) but the values of the two displacements approach equality as  $K_{Na} \longrightarrow \infty$ . The effect of the competitive interaction between sodium and calcium ions is illustrated in Fig.(1).

The effect of the reduction in the sodium concentration on the action potential must now be considered. If it is assumed that the output of acetylcholine is reduced by a constant factor (i.e. independent of the calcium concentration cf. Takeuchi and Takeuchi (1962) and Miledi and Slater (1966) ) and if it is further assumed that the reduction in the action potential is also independent of the calcium concentration, then 'the action potential effect' will modify Fig.(4.1) as shown in Fig.(4.2).

Inspection of Fig(4.2) shows that the output of acetylcholine would be expected to be increased in low calcium concentrations and reduced in high calcium concentrations by a reduction in the sodium concentration. Moreover, in low sodium concentrations the relationship between the output and the log of the calcium concentration would be less steep than at the normal sodium concentration.

Fig. (4.2)

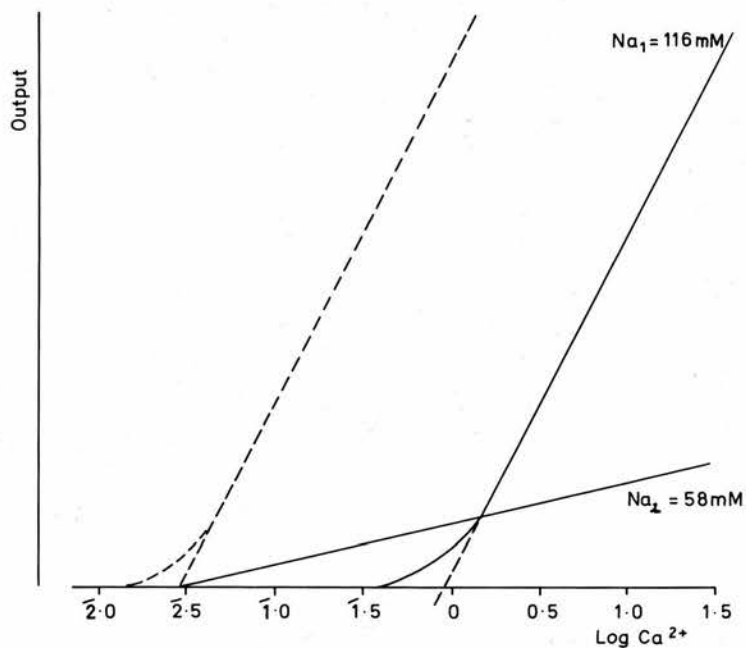


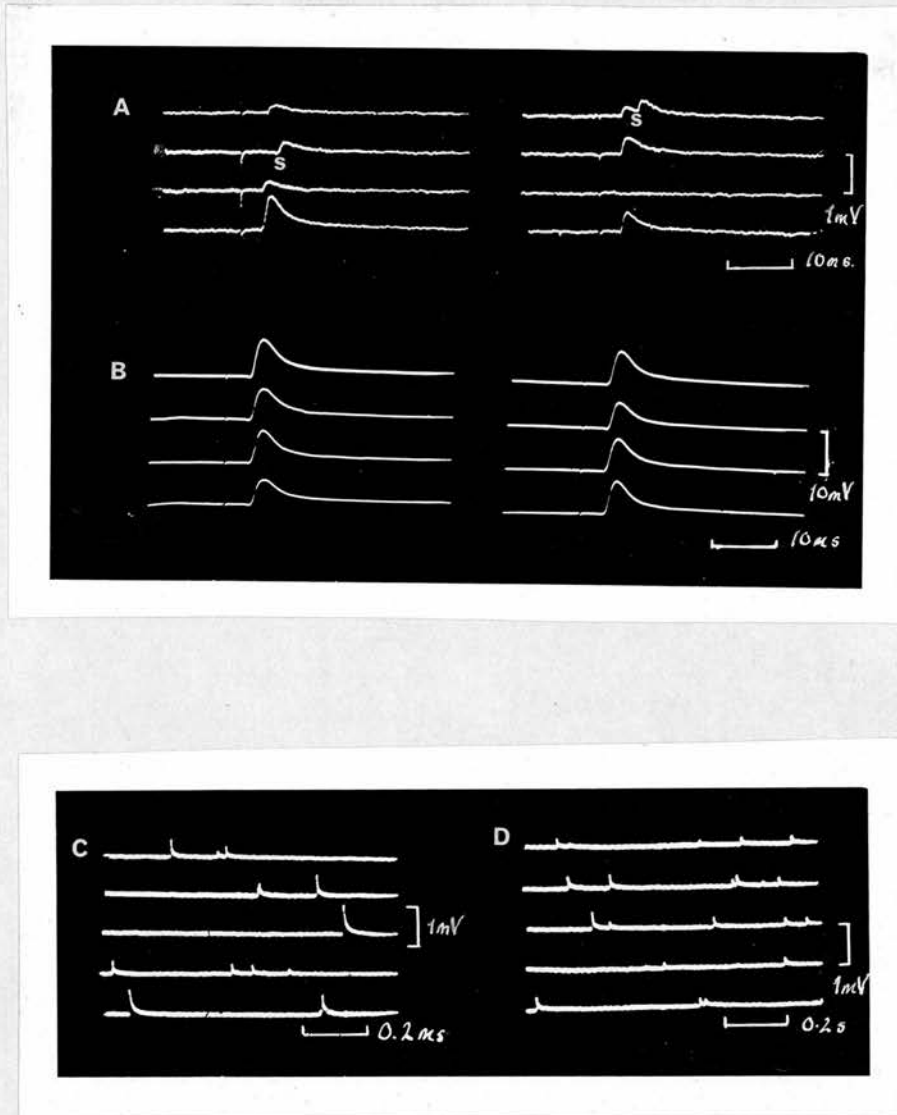
Fig. (4.2) Fig. (4.1) modified to show the combined effects of a shift to the left on the basis of equation (6) and the 'action potential effect' that would be expected as a result of the reduction in the NaCl concentration to 58 mM.



RESULTS - QUANTAL CONTENTI Calcium Deficient Ringer(a) One-eighth normal calcium

Evoked and spontaneous miniature e.p.p.'s were recorded intracellularly from sartorii muscles bathed in Ringer solutions which contained only one-eighth of the normal calcium concentration (0.23mM) and various amounts of the sodium chloride replaced by osmotically equivalent amounts of sucrose. The procedure was to record a series of e.p.p.'s evoked by a 100 successive nerve stimuli at 1 every 3.2 sec and 50 spontaneous miniature e.p.p.'s from the same end-plate immediately before and 5 minutes after the sodium concentration in the bath was altered.

Evoked e.p.p. In every one of the sixteen experiments (Table 4.1 p. 69 ) in which the bathing solution was successfully changed without disturbing the microelectrode the amplitudes of the evoked e.p.p.'s increased dramatically when the sodium concentration was reduced and just as quickly declined when the sodium concentration was restored to normal. For example in the experiment illustrated in Fig. (4.3 p. 47 ) in which the sodium concentration was reduced to one-third, the mean amplitude of the evoked e.p.p.'s increased from 0.27 mV to 6.45 mV.

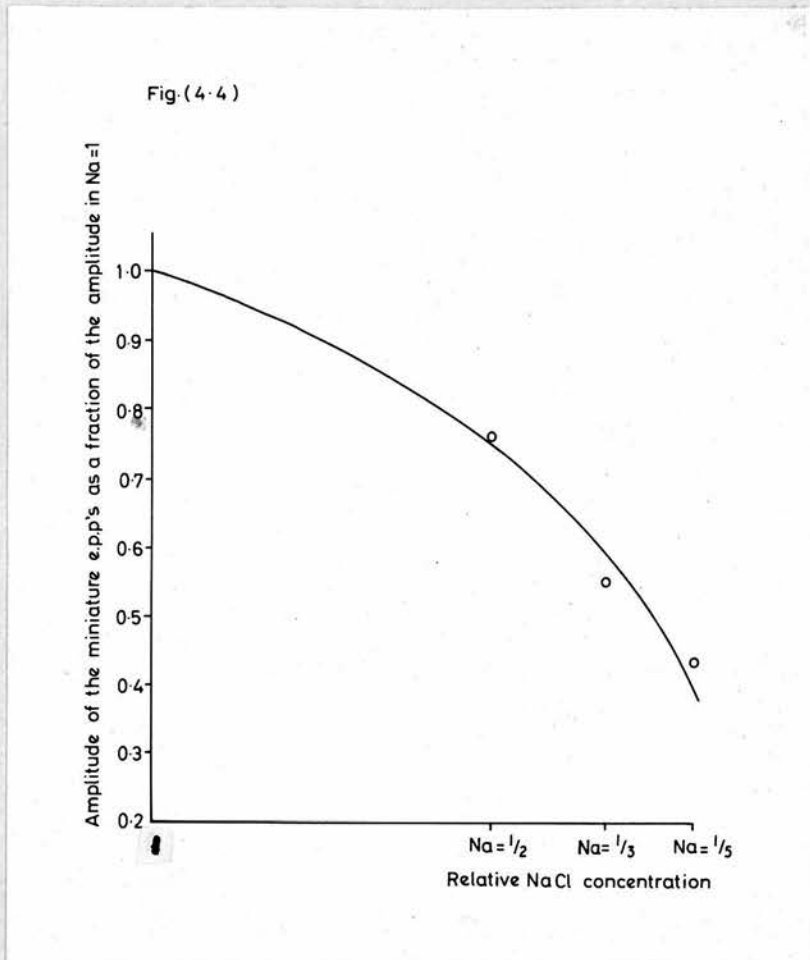


**Fig. (4.3)** Effect of replacing NaCl by sucrose on the evoked and miniature e.p.p.'s in 0.23 mM  $\text{Ca}^{2+}$ . A - Selection of evoked e.p.p.'s in normal NaCl. Mean quantal content per nerve impulse, 0.9. Two stimuli were 'failures'; S indicates probable miniature e.p.p. B - responses recorded at the same end-plate in 1/3 normal NaCl. Note 10-fold change in gain. Mean quantal content 36. C and D - Miniature e.p.p.'s in normal NaCl (C) and 1/3 normal (D).

Miniature e.p.p.'s. As would be expected the amplitudes of the miniature e.p.p.'s decreased when the sodium concentration of the bathing solution was lowered and increased when the normal sodium concentration was restored (Table 4.2 p. 70 ). Fig. (4.4 p. 49) shows that the reduction or increase which occurs in the mean amplitude of the miniature e.p.p.'s when the sodium concentration is altered, is related to the change in the sodium concentration.

The effect on quantal content.

The increase in the amplitudes of the e.p.p.'s was clearly characteristic of an increase in the mean number of quanta released per stimulus since the <sup>increase in the</sup> ~~the~~ mean amplitude of the evoked e.p.p. was accompanied by a decrease in the amplitudes of the miniature e.p.p.'s. It may also be noted that when the sodium concentration was normal failures frequently occurred (i.e. a number of stimuli failed to evoke a response) but in reduced sodium concentrations every stimulus evoked a response. The mean quantal content was calculated as the ratio of the mean amplitude of the evoked e.p.p.'s to the mean of the miniature e.p.p.'s (equations 2 and 6, chapter 3). In sixteen experiments, (Table 4.3 p. 50 ) the mean quantal content of the evoked e.p.p.'s recorded from the same end-plate bathed in two different sodium concentrations, was much larger in the lower sodium concentration.



**Fig. (4.4)** Effect of reduction in NaCl on size of miniature e.p.p.'s. Ratio of mean amplitude in low  $\text{Na}^+$  to mean amplitude in normal  $\text{Na}^+$  is plotted against NaCl concentration. Individual results shown in Table (4.4).

Table (4.3) Summary of Table (4.1). ( $\rightarrow$ ) signifies that in this particular experiment the NaCl concentration was reduced and ( $\leftarrow$ ) that the NaCl concentration was increased.

Expt. No.	Direction of Change	Relative NaCl concentration			
		1	1/2	1/3	1/5
Mean quantal contents					
1	→	0.3	2.2		16.0
2	→	2.2	5.1		
3	→	0.9		14.0	
4	→	2.2		35.0	
5	→	5.0			25.1
6	→	0.8			21.0
7	←	1.4	13.0		
8	←	1.2	14.7		
9	←	4.0		100.0	
10	←	0.9		36.0	
11	←	0.8			13.1
12	←	0.9			52.4
13	→			19.3	56.4
14	→			15.0	28.5
15	←			15.6	43.4
16	←			12.2	33.2



Table (4.4) below shows the average number of quanta released at a number of end-plates bathed in four different sodium concentrations.

TABLE (4.4)

Relative sodium concentration	'Na1'	'Na1/2'	'Na1/3'	'Na1/5'
Average quantal content	2.3	8.0	38	46
S.E. $\pm$	0.5	2.0	10	5
Number of end-plates	(18)	(6)	(12)	(22)

The greater the reduction in the sodium chloride concentration the greater was the reduction in the mean quantal content.

Replacement of sodium chloride by glycine.

Table (4.5 p. 52) shows the results from four experiments in which the sodium chloride replacement was glycine instead of sucrose. All the solutions contained an additional 8ml of phosphate buffer per litre in order to avoid alterations in the pH when sodium chloride was replaced by glycine. A reduction in the sodium concentration from normal to one-third normal caused the average quantal content in the four experiments to increase by a factor of about 12 from 2.1 to 25.9.



Table (4.5) The results of 4 experiments which show the effect of replacing NaCl by isotonic glycine. The calcium concentration was 0.23 mM and Na = 1 corresponds to 115.6 mM. In experiment (4) \* signifies that the replacement was sucrose not glycine.

Expt. No.	Relative sodium conc.	Mean amplitude of evoked e.p.p.'s in mV and SD	Mean amplitude of the miniature e.p.p.'s in mV.	Quantal Content $\sqrt[3]{m_1 m_2}$		
				$m_1$	$m_2$	$m_3$
1	1	1.34 $\pm$ 0.80	0.39	3.4		
	1/3	6.76 $\pm$ 1.29	0.28	24.1	27.5	25.2
2	1	0.58 $\pm$ 0.71	0.62	1.0		
	1/3	5.79 $\pm$ 1.32	0.44	13.1	19.2	14.8
3	1	0.81 $\pm$ 0.59	0.43	1.9		
	1/3	9.57 $\pm$ 1.10	0.30	32.5	72.4	41.6
4	1	1.24 $\pm$ 0.78	0.56	2.2		
	1/3	6.74 $\pm$ 1.59	0.45	16.0	43.1	22.2
	*1/3	6.39 $\pm$ 0.98	0.45	16.4	45.8	23.1

The quantal contents in normal sodium were calculated as before from the ratio of the mean amplitude of the e.p.p.'s to the mean amplitudes of the miniature e.p.p.'s. When two-thirds of the sodium was replaced by glycine the mean amplitude of the e.p.p.'s was greater than 5 mV; instead of correcting the amplitudes of the e.p.p.'s for "non-linear summation" the best estimate of the quantal content was taken to be  $m_3$ , the weighted mean of the two estimates  $m_1$  (the ratio of the mean amplitudes of the e.p.p.'s and the miniature e.p.p.'s) and  $m_2$  (the ratio of the square of the mean amplitude of the e.p.p.'s to the variance of the e.p.p. amplitudes) (see chapter 3, equations(2), (4) and (9) ). In the final experiment (4) shown in table (4.5) it was found that the quantal content was unaltered when the second solution was exchanged for a third in which the sodium replacement was sucrose instead of glycine.

#### Variability.

The increase or decrease in quantal content of the e.p.p. produced by any particular alteration of the sodium concentration was extremely variable. For example when half the sodium chloride was replaced by sucrose the output of transmitter was 3.2 to 11.6 times greater than in normal sodium chloride and when two-thirds and four-fifths were replaced, the output increased from 15.7 to 31.8 and 4.2 to 53 times respectively.

The results were obtained from a rather selected population of end-plates as it was only possible to study selected end-plates from suitable muscles. For example a number of muscles were not used because neuromuscular block failed to develop even after prolonged immersion in calcium deficient Ringer solution. Others proved unsuitable because they twitched in response to nerve stimulation after quite small reductions in the sodium concentration. Kita (1966) has shown that reduction of the sodium concentration below 50mM causes a reduction in the critical threshold to which a muscle fibre must be depolarized before a propagated spike potential is initiated.

There is no doubt, however, that in one-eighth calcium a reduction in the extracellular sodium chloride concentration causes an increase in the output of transmitter per nerve impulse and that the size of the increase is related to the extent of the alteration in the sodium chloride concentration.

(b) One-half normal calcium.

Quantal contents were determined in 15 experiments (equation 6, chapter 3) when the sodium concentration was one-fifth of the normal and the calcium concentration was one-half normal (Table 4.6). The average value was  $99 \pm 11$  S.E.

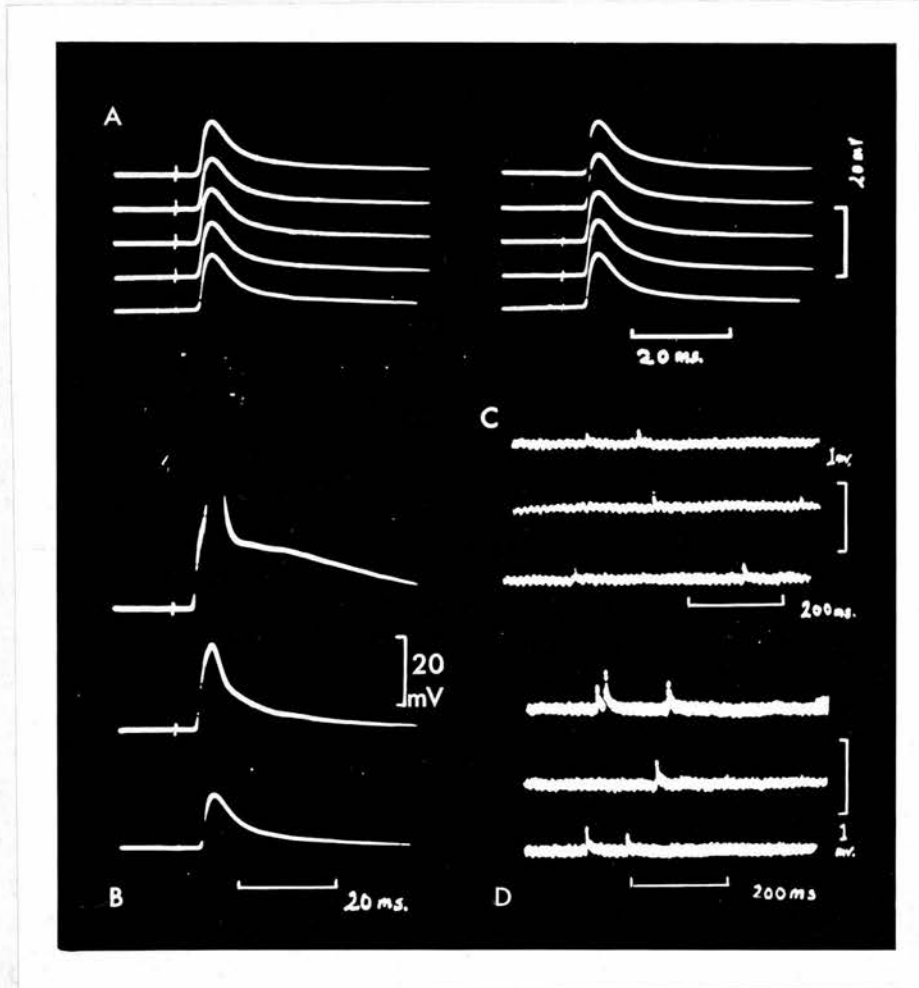
Table (4.6) The results of 15 experiments in which the quantal contents were determined in solutions containing 23.1 mM NaCl and 0.9 mM  $\text{Ca}^{2+}$ . The mean quantal content was  $99 \pm 11$  S.E.

Expt. No.	Mean amplitude of e.p.p.'s (corrected for non linear summation) in mV and SD		Mean amplitude of miniature e.p.p.'s	Quantal Content $m'$
1	9.28	0.86	0.10	94
2	22.56	1.98	0.22	104
3	11.83	0.84	0.08	148
4	9.69	0.31	0.08	121
5	3.85	0.23	0.07	60
6	4.82	0.25	0.10	50
7	6.25	0.68	0.08	75
8	13.04	-	0.06	204
9	9.02	0.49	0.14	66
10	12.47	1.26	0.16	76
11	9.30	0.81	0.09	101
12	7.87	0.88	0.11	70
13	24.19	-	0.21	114
14	5.73	0.71	0.11	51
15	23.70	1.59	0.16	148



It was not possible as a routine to determine the quantal content directly in normal sodium and one-half calcium because nerve stimulation gave rise to action potentials and muscle twitches. However, in two experiments an attempt was made to follow the change in quantal content at one-half of the normal calcium concentration when the sodium concentration was increased from one-fifth to normal. In these experiments the microelectrode was not dislodged by the muscle twitch and it was possible to record miniature e.p.p.'s in both sodium concentrations. In one of the experiments, illustrated in Fig. (4.5 p. 57 ) the amplitude of the e.p.p. increased 1.7 times from 23.7 to 40.7mV (corrected for non-linear summation) when the sodium chloride concentration was restored to normal from one-fifth normal. In the other experiment the change was 1.9 times from 7.9 to 14.9mV. The corresponding increase in the mean amplitude of the miniature e.p.p.'s was 1.5 times in both experiments. The alterations in the mean amplitudes of the e.p.p.'s were equivalent to an increase in quantal content from 148 in low sodium to 171 in normal sodium in the first experiment and from 70 to 93 in the other, but these differences cannot be regarded as significant.

An approximate value for the quantal content in one-half the normal calcium concentration and the normal sodium concentration can also be derived from the published



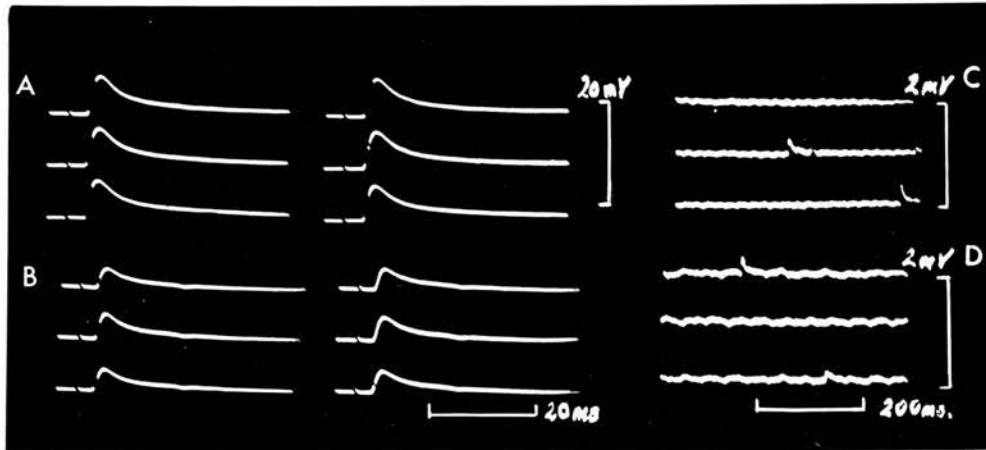
**Fig. (4.5)** Effect of replacing sucrose by NaCl on evoked and miniature e.p.p.'s in 0.9 mM  $\text{Ca}^{2+}$ .

A - Evoked e.p.p.'s in 1/5 normal NaCl. Mean quantal content, 148. B - E.p.p.'s from same end-plate as concentration of NaCl was increased to normal, reading from bottom to top. In normal NaCl e.p.p. initiated action potential. Quantal content about 171 quanta. C and D - Miniature e.p.p.'s in (C) 1/5 NaCl, 4/5 sucrose (D) normal NaCl.

results of Martin (1955) and Jenkinson (1957). Jenkinson has shown that the mean amplitude of the externally recorded e.p.p. decreases four fold when the calcium concentration is reduced from twice normal to one-half normal: Martin estimates a quantal content of 240 for the end-plate step of an action potential recorded in normal sodium in twice the normal calcium concentration. Thus one value for the quantal content is 60; this is somewhat smaller than 99, the value obtained when the sodium concentration was one-fifth normal and the calcium concentration one-half normal. However, it is evident that a reduction in the sodium concentration from normal to one-fifth has no marked effect on the release of transmitter when the calcium concentration is maintained at one-half normal.

concentration

It is also clear that when the sodium <sup>concentration</sup> is reduced to one-fifth of the normal, the quantal content is rather insensitive to changes in the calcium concentration since an increase in the calcium concentration from one-eighth to one-half normal increased the quantal content by a factor of only about 2 (from 46 to 99 quanta). In the experiment shown in Fig. (4.6 p. 59 ) the effect of an increase in calcium concentration at the same end-plate was determined. Two sequences of evoked and spontaneous e.p.p.'s, recorded before and after the calcium concentration was increased from one-eighth to one-half of the normal, demonstrate that a four fold change in the



**Fig. (4.6)** Effect of an increase in  $Ca^{2+}$  concentration on evoked and miniature e.p.p.'s in 1/5 NaCl and 4/5 sucrose. A - Evoked e.p.p.'s in 0.9 mM  $Ca^{2+}$ . Mean quantal content 94. B - Evoked e.p.p.'s from the same end-plate in 0.23 mM  $Ca^{2+}$ . Mean quantal content 43. Miniature e.p.p.'s before (C) and after (D) change in  $Ca^{2+}$  concentration.

calcium concentration when the sodium concentration is one-fifth normal only causes a two fold change in the quantal content, from 43 to 94.

When the extracellular sodium concentration was normal Jenkinson (1957) found that the amplitude of the e.p.p. in curarized muscle increased 5.5 times when the calcium concentration was increased two fold from one-quarter to one-half normal.

#### EXPERIMENTS IN SOLUTIONS CONTAINING TUBOCURARINE

In experiments where the calcium concentration was less than normal the output of transmitter responded to changes in the sodium concentration in a way which was predictable qualitatively from the hypothesis illustrated by Fig(4.2 p. 45.) A decrease in the sodium concentration increased the quantal content when the calcium concentration was one-eighth normal and caused no demonstrable effect when the calcium concentration was one-half normal. In one-fifth sodium the quantal content was relatively insensitive to a four fold increase in the calcium concentration.

This finding was confirmed in six experiments (Table 4.7 p. 61 ) in which the e.p.p. was recorded in both one-eighth and one-half the normal calcium from the same end-plate bathed in solutions which contained one-fifth of the normal sodium concentration and  $0.3-1.0 \times 10^{-6}$  M tubocurarine. The alteration in the calcium concentration



Table (4.7) The results of experiments at 6 end-plates to show the effect of an increase in the  $\text{Ca}^{2+}$  concentration from 1/8 to 1/2 normal when NaCl is 1/5 normal. The amplitude of the e.p.p.'s in experiments 28 and 29 were corrected for 'non-linear summation'. In the other four experiments both solutions contained the same tubocurarine concentration ( $0.3-1.0 \times 10^{-6}$  M).

Experiment No.	End-plate potential in mV in a $\text{Ca}^{2+}$ concentration of $\text{Ca}^{2+}$ 1/8 $\text{Ca}^{2+}$ 1/2	
28	4.20	9.28
29	15.0	22.56
30	2.14	3.85
31	1.32	2.37
32	1.15	2.96
33	1.22	2.83

led to an increase in the average amplitude of the e.p.p.'s in the six experiments, from 4.17 to 7.31mV, a factor of 1.75.

## II Normal or twice normal calcium

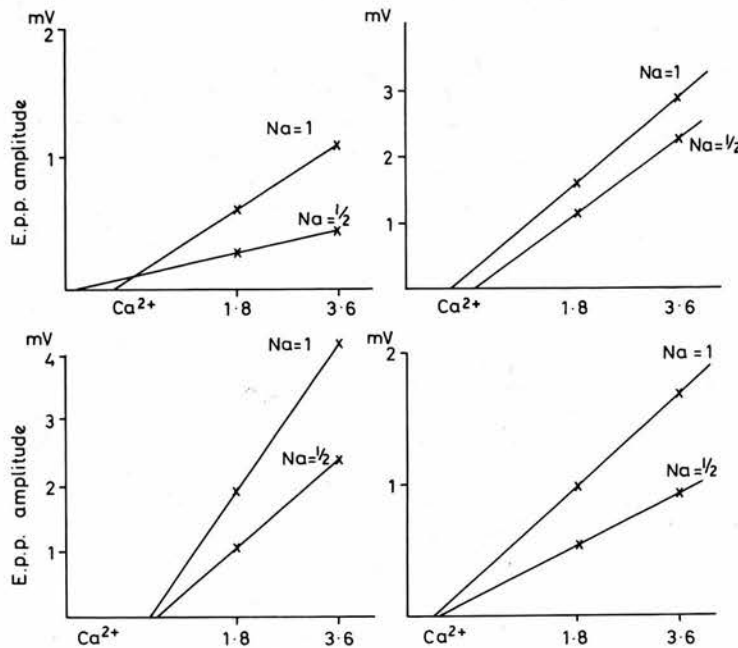
At higher calcium concentrations, the hypothesis predicted that a reduction in the sodium concentration would reduce the output of transmitter to a small proportion of that in normal sodium. For each increment in the calcium concentration the output in low sodium would be a progressively smaller proportion of the output in normal sodium (see Fig 4.2 p. 45 ).

In the higher calcium concentrations, the quantal content cannot be measured directly and changes in the output of transmitter must be inferred from the way in which the amplitudes of the e.p.p.'s in curarized muscle alter. Unfortunately, a decrease in the amplitude of the curarized e.p.p. will occur when the sodium concentration is reduced even if the output of transmitter remains constant. The reduction in amplitude is due to (a) a decrease in the equilibrium potential and (b) an increase in the effectiveness of tubocurarine in solutions containing reduced sodium concentrations (Jenkinson, 1960). The size of the decrease in amplitude due to the reduction in the equilibrium potential can be estimated from Fig. (4.4 p. 49) which shows the relationship between the reduction in the amplitude of the miniature e.p.p.'s and

the change in the extracellular sodium concentration. On the other hand there is no simple way of correcting the amplitude of the e.p.p.'s recorded in a reduced sodium concentration for the increase in the level of curarization which occurs. An additional complication is that the affinity of tubocurarine for the acetylcholine receptors appears to be reduced (at least in normal sodium concentration) when the calcium concentration is increased (Jenkinson, 1960). Nevertheless, the experiments described below suggest that the reduction in the output of transmitter when the sodium chloride concentration is reduced is small or does not occur at high calcium concentrations.

The mean amplitudes of e.p.p.'s recorded from the same end-plate of a curarized muscle bathed in two solutions which contained the normal sodium concentration and either the normal or twice the normal calcium concentration were compared with the mean amplitude of the e.p.p.'s recorded in the presence of two complementary solutions which contained the same tubocurarine and calcium concentrations but only half the sodium chloride concentration (see chapter 3). From the results of each experiment (see Fig. 4.7) the ratio of the mean amplitude of the e.p.p.'s recorded in one half sodium to the mean amplitude in normal sodium was calculated at each calcium concentration.

Fig. (4.7)



**Fig. (4.7)** The results of four experiments in which the amplitude of the e.p.p. was recorded at the same end-plate in normal NaCl and one-half normal NaCl concentration at two different  $\text{Ca}^{2+}$  concentrations, 1.8 and 3.6 mM. Solutions contained  $3 \times 10^{-8}$  M tubocurarine.

The two ratios obtained in each individual experiment are shown in Table (4.8).

TABLE (4.8)

Experiment No.	Relative Calcium Concentration	
	Normal Ca	2 x Normal Ca
1	0.46	0.49
2	0.77	0.78
3	0.56	0.58
4	0.56	0.56

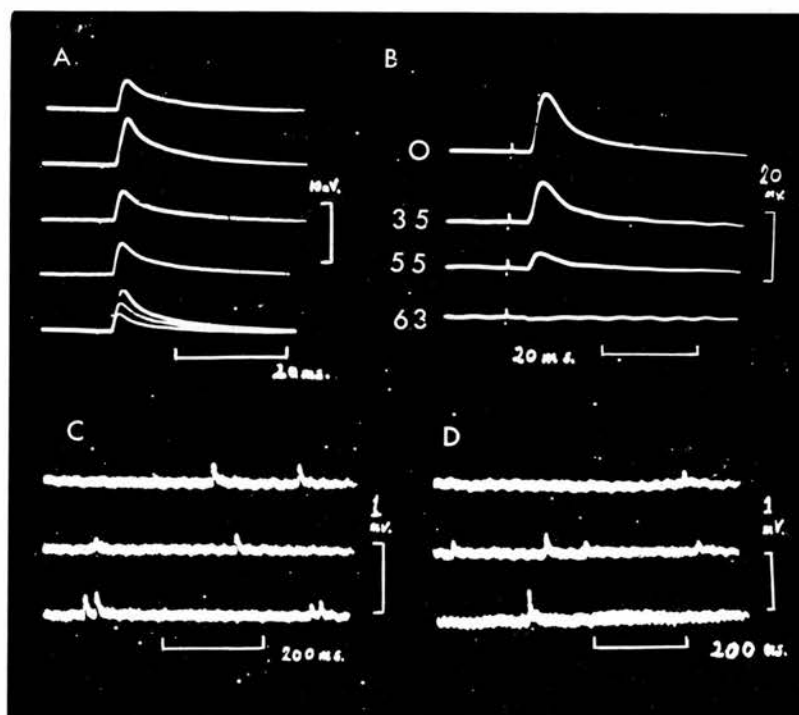
In normal calcium, the mean amplitudes of the e.p.p.'s recorded in one half the normal sodium concentration were 0.46 to 0.77 times the amplitude in normal sodium. The greater part of this reduction in the mean amplitude of the e.p.p.'s in low sodium does not represent a decrease in the output of transmitter. A reduction to 0.76 of the mean amplitude in normal sodium can be accounted for by the reduction in the equilibrium potential alone, and a further reduction would be expected as a result of the increase in curarization which occurs when the sodium concentration is reduced. Even more significant is that in no individual experiment shown in table (4.8) is the ratio of the mean amplitude of the e.p.p.'s in one-half sodium to the mean amplitude in



normal sodium greater at normal calcium than it is at 2 x normal calcium. In other words even allowing that the output in normal calcium is reduced when the sodium concentration is reduced, the output in twice the normal calcium concentration must be reduced to a lesser degree. It also follows that the increase in the output of transmitter in one-half the normal sodium concentration which occurs in response to a change in the calcium concentration from normal to twice normal cannot be smaller than that which occurs in normal sodium. These results are therefore difficult to reconcile with the simple hypothesis outlined in the introduction.

#### Nerve Block

The output of transmitter in calcium concentrations greater than one half normal could not be investigated when four-fifths of the sodium chloride was replaced by sucrose, because of the occurrence of varying degrees of nerve block in the majority of the nerve terminals. The intermittent onset of partial nerve block which occurred during the recording of a series of e.p.p.'s is shown in Fig. (4.8 p. 67 ). The majority of the e.p.p.'s recorded had a mean amplitude of 7.0mV, and only the amplitude of one e.p.p. in 20 would have been expected to fall outside the range 5 - 9mV. Intermittently, however, the random pattern of the fluctuation of the amplitudes of the e.p.p.'s was interrupted and several consecutive e.p.p.'s with amplitudes less than 5mV appeared before the original pattern was resumed. This



**Fig. (4.8)** Intracellular records from two end-plates at which nerve block occurred. A - evoked e.p.p.'s recorded in normal NaCl and 0.9 mM  $\text{Ca}^{2+}$ . Majority of e.p.p.'s recorded were like upper four records with mean of 7 mV and fluctuating between 5 and 9 mV. Intermittently, fluctuation greatly increased as shown by the three superimposed traces. B - Uppermost e.p.p. in 1/5 normal NaCl and 0.9 mM  $\text{Ca}^{2+}$  subsequent e.p.p.'s recorded 35, 55 and 63 sec after sufficient isotonic  $\text{CaCl}_2$  added to bath to raise  $\text{Ca}^{2+}$  concentration to 1.8 mM. C and D show spontaneous end-plate potentials recorded at the same end-plate before (C) and after (D) the addition of isotonic  $\text{CaCl}_2$ .

effect was probably due to a failure of the action potential to propagate into all the branches of the nerve terminal. Intermittent partial nerve block often heralded the appearance of complete nerve block.

The onset of complete nerve block is shown in Fig. (4.8 p. 67 ) by a series of records which show the rapid decline of the e.p.p. amplitude to zero in the 65 sec after the calcium concentration was raised from one half normal to normal. Miniature e.p.p.'s recorded before and after the appearance of complete nerve block show that there had been no change in the post-synaptic response to individual quanta.

On occasion, every nerve terminal appeared to be blocked as evoked e.p.p.'s could not be recorded from the muscle, although end-plate regions could be identified by the presence of spontaneous miniature e.p.p.'s. When the sodium concentration of the bath was increased or the calcium concentration decreased e.p.p.'s could then be evoked.

Table (4.1) The results of 16 experiments which show the effect of changing the NaCl concentration by replacement with isotonic sucrose. The calcium concentration was one eighth normal (0.23 mM) and Na = 1 corresponds to 115.6 mM NaCl. End-plate potentials greater than 2 mV were corrected for 'non linear summation'.

Expt. No.	Relative NaCl Conc.	Mean amplitude of evoked e.p.p.'s in mV and SD	Mean amplitude of the miniature e.p.p.'s in mV	Quantal Content
1	1	0.12 <sup>±</sup> 0.23	0.41	0.3
	1/2	0.61 <sup>±</sup> 0.42	0.27	2.2
	1/5	1.53 <sup>±</sup> 0.58	0.12	13.0
2	1	0.50 <sup>±</sup> 0.34	0.25	2.0
	1/2	1.16 <sup>±</sup> 0.73	0.23	5.1
3	1	0.27 <sup>±</sup> 0.24	0.32	0.9
	1/3	7.39 <sup>±</sup> 1.11	0.20	36.0
4	1	0.63 <sup>±</sup> 0.47	0.29	2.2
	1/3	7.78 <sup>±</sup> 1.07	0.22	35.0
5	1	1.38 <sup>±</sup> 0.63	0.27	5.0
	1/5	4.30 <sup>±</sup> 0.55	0.17	25.1
6	1	0.31 <sup>±</sup> 0.36	0.40	0.8
	1/5	5.90 <sup>±</sup> 1.73	0.28	21.0
7	1/2	1.18 <sup>±</sup> 0.37	0.09	13.0
	1	0.16 <sup>±</sup> 0.13	0.12	1.4

Table (4.1) (continued)

Expt. No.	Relative NaCl Conc.	Mean amplitude of evoked e.p.p.'s in mV and SD	Mean amplitude of the miniature e.p.p.'s in mV	Quantal Content
8	1/2	$1.91 \pm 0.42$	0.13	14.7
	1	$0.11 \pm 0.10$	0.09	1.2
9	1/3	$15.36 \pm 1.18$	0.15	100.0
	1	$1.56 \pm 0.73$	0.41	4.0
10	1/3	$4.93 \pm 0.98$	0.35	13.1
	1	$0.55 \pm 0.44$	0.58	0.9
11	1/5	$1.59 \pm 0.18$	0.12	13.1
	1	$0.14 \pm 0.13$	0.17	0.8
12	1/5	$7.0 \pm 1.23$	0.13	52.0
	1	$0.21 \pm 0.21$	0.22	0.9
13	1/3	$2.13 \pm 0.58$	0.11	13.3
	1/5	$4.45 \pm 0.47$	0.08	56.4
14	1/3	$3.10 \pm 0.85$	0.20	15.0
	1/5	$4.81 \pm 0.63$	0.17	28.5
15	1/5	$7.54 \pm 1.22$	0.17	43.3
	1/3	$3.61 \pm 0.86$	0.23	15.6
16	1/5	$6.24 \pm 1.07$	0.19	33.2
	1/3	$3.08 \pm 0.81$	0.25	12.2



**Table (4.2)** Effect of reduction in NaCl on the mean amplitude of miniature e.p.p.'s. The results of experiments 1 - 16 have already appeared in Table (4.1). The  $\text{Ca}^{2+}$  concentration was 0.23 mM except in experiments 22 - 27 when it was 1.8 mM. The solutions in experiments 22 - 27 also contained prostigmine  $4.45 \times 10^{-8}$  M. ( $\rightarrow$ ) signifies that in this particular experiment the  $\text{Na}^+$  concentration was reduced and ( $\leftarrow$ ) that the  $\text{Na}^+$  concentration was increased. (See Fig (4.4) ).

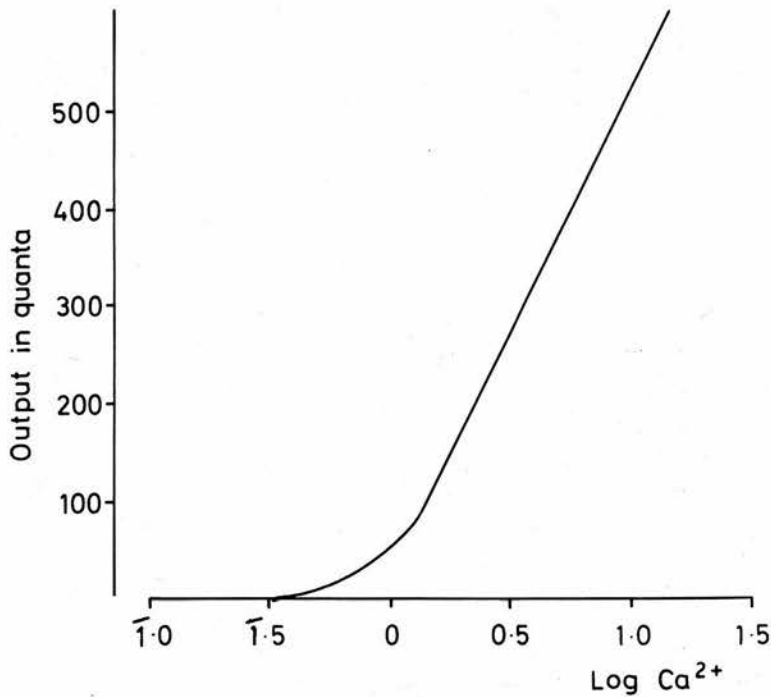
Expt. No.	Direction of Change	Relative NaCl concentration			
		1	1/2	1/3	1/5
		mean amplitude of m.e.p.p. (mV)			
1	→	0.413	0.273		0.118
2	→	0.251	0.229		
17	→	0.310	0.270		
18	→	0.325	0.210		
3	→	0.576		0.347	
4	→	0.289		0.222	
9	←	0.406		0.153	
10	←	0.316		0.204	
20	←	0.482		0.218	
5	→	0.274			0.171
6	→	0.399			0.279
19	→	0.218			0.150
11	←	0.174			0.116
12	←	0.220			0.134
21	←	0.161			0.113
22	→	0.488			0.182
23	→	0.545			0.247
24	→	0.703			0.275
25	←	0.497			0.212
26	←	0.583			0.308
27	←	0.804			0.455

### DISCUSSION

The results obtained in subnormal calcium concentrations appear qualitatively to be in agreement with the hypothesis outlined in the introduction. Thus a reduction in the sodium chloride concentration has been shown to increase the quantal content when the calcium concentration is one-eighth of the normal and to have no demonstrable effect when the calcium concentration is half normal. The output of transmitter when the sodium concentration is one-fifth of the normal was found to be rather insensitive to a fourfold increase in the calcium concentration when compared with the effect in normal sodium. Quantitatively, as will be shown below, however, the results do not support the hypothesis. In high calcium concentrations the results are not even in qualitative agreement with the hypothesis.

Nevertheless it seems worth exploring the discrepancies between the results and the hypothesis. For this purpose, the results published by Jenkinson (1957) will be taken as representative of the variation in the output of acetylcholine with calcium at the normal sodium concentration (see Fig. 4, 9). To transform the amplitudes of the e.p.p.'s into quantal content, the quantal content in normal sodium and one-half normal calcium was assumed to be 100 quanta (see p. 54 ). The results will be analysed in terms of the detailed

Fig. (4.9)



**Fig. (4.9)** The relationship between the output of transmitter in quanta and the log of the calcium concentration. The curve was redrawn from Jenkinson (1957) on the assumption that the average quantal content in normal sodium at  $\text{Ca}^{2+} = 0.9 \text{ mM}$  is 100 quanta.

interaction between sodium and calcium ions treated in the theoretical introduction to this chapter. The two essential features of the hypothesis are (a) that there is a parallel shift of the output/log calcium curve and (b) that the 'action potential effect' consists of a reduction of the output of acetylcholine by a factor which is constant for any particular reduction in sodium chloride. The shift of the output curve has thus far been considered to be the result of the reduction in sodium concentration and to be the consequence of certain specific forms of interaction between sodium and calcium ions. However, the analysis would not differ in principle if a specific interaction between sodium and calcium ions of different forms occurred or if the interaction were non-specific. If a non-specific interaction occurred, for example, the output/log calcium curve would still be expected to be subjected to a parallel shift when the sodium chloride was reduced, since in any particular sodium chloride concentration the ionic strength remained constant.

Subnormal calcium concentration. In terms of the first form of the hypothesis calcium and sodium ions compete for a single receptor by an ion exchange mechanism (equation 6 p. 41). If the amplitude of the presynaptic action potential was unaffected by a reduction in the sodium concentration and if it is assumed that the output of transmitter is unaltered when the ratio between the

calcium concentration and the square of the sodium concentration is kept constant, the quantal content, when the calcium concentration is one-eighth normal, would be expected to be 100, 274 and 500 when the sodium concentration is one-half, one-third and one-fifth of normal respectively. If the hypothesis is correct the ratio of the expected to the observed quantal contents should be a measure of the reduction in output attributable to the effect of the reduction in the amplitude of the pre-synaptic action potential.

In table (4.9) below, the quantal contents in four experiments when the sodium concentration was changed between one-third and one-fifth and the calcium concentration was one-eighth normal, are compared with the 'expected values'.

TABLE (4.9)

Comparison between 'expected' and observed quantal contents in 4 single experiments

Expt. No.	$1/5 \text{ Na}^+$			$1/3 \text{ Na}^+$		
	Expected quantal content	Observed quantal content	Ratio of 'expected' to observed	Expected quantal content	Observed quantal content	Ratio of 'expected' to observed
13	500	56	8.9	274	19	14.5
14	500	29	17.0	274	15	18.3
15	500	40	12.5	274	17	16.1
16	500	33	15.1	274	12	22.8



In all four experiments the ratio between the expected value and the observed is greater in the larger of the sodium concentrations. This is incompatible with the idea that the greater the reduction in the amplitude of the presynaptic action potential the greater will be the ratio between the 'expected' and the observed quantal content. The ratio between the 'expected' and observed quantal content is also much smaller than expected on the basis of Liley's (1956c) hypothesis. A reduction in the sodium concentration to a fifth or a third would reduce the amplitude of an action potential in an axon from say 100mV to 60mV or 73mV. On the assumption that for every 10mV the action potential depolarizes the presynaptic nerve terminal the probability of quantal release rises a 100 fold, 60mV and 73mV action potentials will only release  $1/115$  or  $1/29$  respectively of the amount of transmitter released by a 100mV action potential (see appendix to chapter 1 p. 17 ).

The ratios (Table 4.10) between the 'expected' and the average quantal content observed in a number of experiments were equally unfavourable to the hypothesis, since the 'action potential effect' appears to be greater in one-half normal sodium, for example, than in one-fifth normal sodium.

TABLE (4.10)

Comparison between 'expected' and observed  
average quantal contents

Relative sodium and calcium concentrations	'Expected' quantal content	Average observed quantal content	Number of experiments	Ratio of 'expected' to observed
1/5Na 1/8Ca	500	46	22	10.8
1/5Na 1/2Ca	800	96	15	7.8
1/3Na 1/8Ca	274	38	12	7.25
1/3Na 1/8Ca	100	8	6	12.5

One objection to this form of analysis is that the 'expected values' are rather speculative. Although the absolute values of the quantal content may not be correct this should not matter since provided the slope of the output/log calcium curve is correct the relative values of the ratio will not be affected.

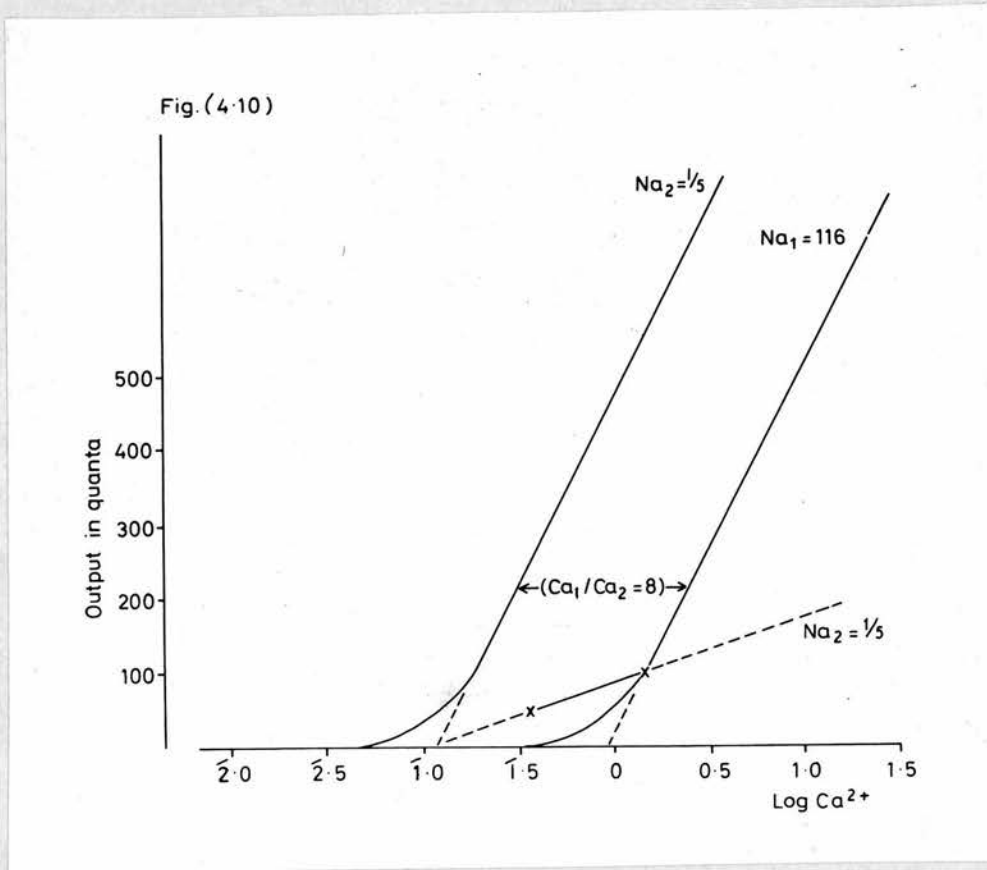
Inspection of Table (4.10) reveals a further discrepancy in that the ratio of the 'expected' to the observed quantal content for a constant sodium concentration decreased as the calcium concentration increased instead of remaining constant.

In summary, the output of acetylcholine does not follow the model based on an ionic exchange type of competition between calcium and sodium ions.

The results may now be considered in terms of equation (11) which is based on the idea that all the receptor sites are <sup>not</sup> occupied by either sodium or calcium ions. To determine a value of the postulated constant  $K_{Na}$  it is again necessary to assume that the 'action potential effect' is independent of calcium concentration. If the quantal contents are known for two different calcium concentrations at the same sodium concentration, reference to Fig. (4.2 p. 45) shows that the 'action potential effect' is given by the ratio of the slope of the output/log calcium curve in normal sodium to the slope of the curve in the reduced sodium concentration.  $K_{Na}$  may be derived from the distance between the points where the two extrapolations of the linear portions of the two curves intercept the log. calcium axis. This distance is equal to

$$\frac{1 + K_{Na} [Na_1]^2}{1 + K_{Na} [Na_2]^2} \dots\dots\dots(14)$$

In Fig. (4.10) the output/log calcium curve for one-fifth sodium was drawn from the quantal contents of 46 and 99 observed in one-eighth and one-half normal calcium. The ratio of the slope of the output/log calcium curve in normal sodium to the slope of the curve in one-fifth sodium was 5.6 and  $K_{Na}$  was calculated to be 770, the distance between the intercepts of the curves being equivalent to an eight fold change in the calcium



**Fig. (4.10)** Comparison between the slope of the observed output/log calcium curve in 23.1 mM NaCl (A) and the slope of the curve in 115.6 mM NaCl (B) and the extrapolation necessary to determine  $[Ca_1] / [Ca_2]$ . (Equation 11 chapter 4).

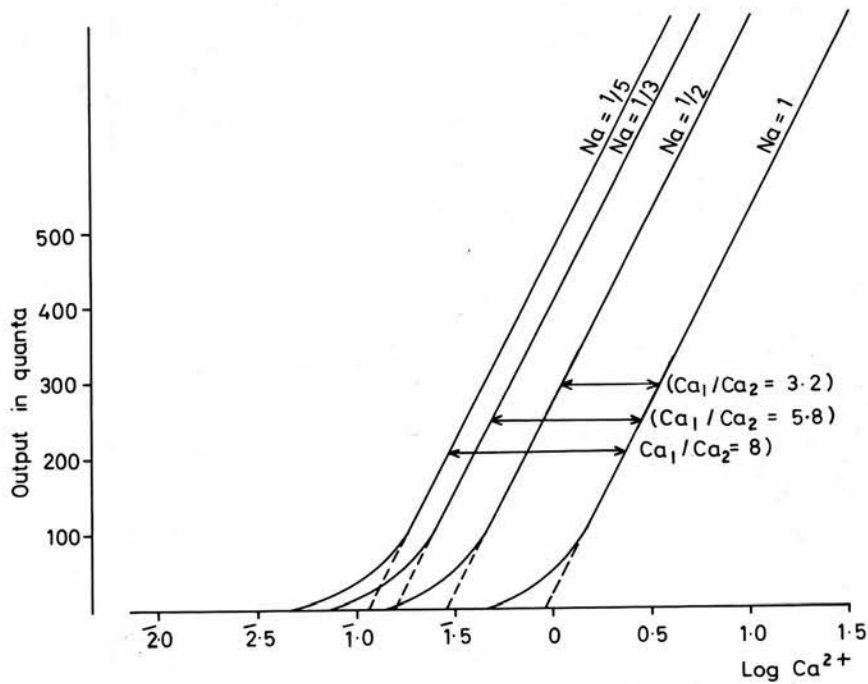
concentration. By substitution of the value of 770 in expression (14) for  $K_{Na}$ , the distance between the intercepts of the output/log calcium curves in sodium one-half and one-third from that in normal sodium, was calculated to be equivalent to a 3.2 and 5.8 fold change in the calcium concentration.

In Fig. (4.11) the appropriate output/log calcium curves have been drawn for the three reduced sodium concentrations without taking into account the effect of a reduction in sodium chloride concentration on the amplitude of the presynaptic action potential. At one-eighth calcium the quantal contents would be expected to be 256, 180 and 50 when the sodium is reduced to one-fifth, one-third and one-half, if the 'action potential effect' is ignored.

In Table (4.11) below, the expected values are compared with the average quantal contents obtained in a number of experiments.



Fig (4-11)



**Fig. (4.11)** The output/log calcium curves that might be expected in NaCl concentrations of  $1/2$ ,  $1/3$  and  $1/5$  normal if  $K_{\text{Na}}$  in equation (14) (p. 77 ) was 770 and the reduction in the NaCl concentration had no effect on the amplitude of the presynaptic action potential.

TABLE (4.11)

Comparison between 'expected' and observed  
average quantal contents

Relative Na <sup>+</sup> and Ca <sup>2+</sup> concentration	'Expected' quantal content	Observed quantal content	Ratio of 'expected' to observed
1/5 Na 1/2 Ca	554	99	5.6
1/5 Na 1/8 Ca	256	46	5.6
1/3 Na 1/8 Ca	180	38	4.8
1/2 Na 1/8 Ca	50	8	6.3

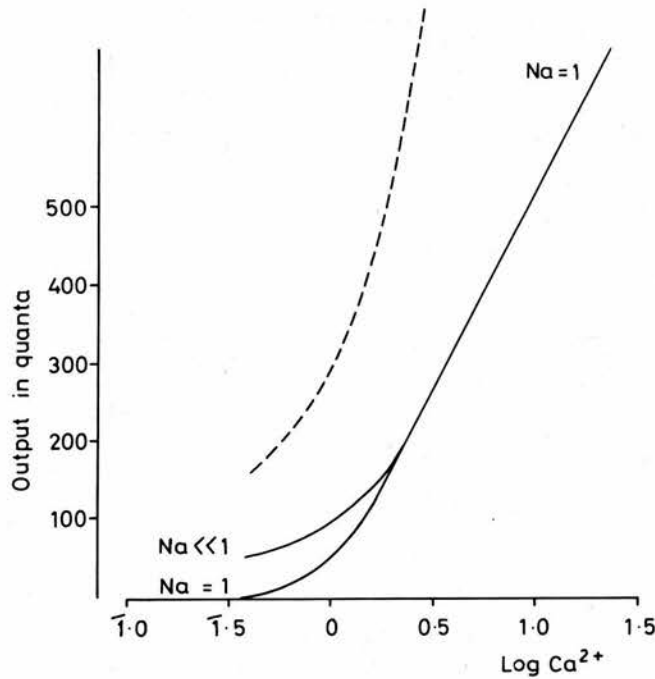
Since the ratio of the 'expected' to the observed quantal content i.e. the magnitude of the 'action potential effect' is much greater when sodium chloride is reduced to one-half than when it was reduced to one-fifth normal, the results are again discordant with the hypothesis. By itself this discrepancy would not challenge the hypothesis since the value of 8 quanta for one-half sodium is the mean of 6 results and the standard error is 2; however, the ratios resulting from a comparison of these expected results and the observed quantal contents in one-fifth and one-third sodium chloride shown for four single experiments in Table (4.9) would also demand that the action potential effect was greater for the smaller reduction in the sodium chloride concentration.

### High calcium concentrations.

As was pointed out in the results there is no evidence to support the view that at a high calcium concentration a reduction in the sodium chloride concentration causes a decrease in the output of transmitter. Furthermore, the extrapolated output/log calcium curves in one-half and in normal sodium in all four experiments, as is shown in Fig. (4.7 p. 64 ) intercept the log calcium axis at almost the same point. If the amplitude of the e.p.p. in one particular sodium concentration and a constant concentration of tubocurarine is proportional to the quantal content, the distance between the intercepts of the two curves should still be, according to the hypothesis, a measure of the competition between sodium and calcium. If the view that there is competition between calcium and sodium is to be preserved it must be postulated that the 'action potential effect' is reduced as the calcium concentration is increased.

It is ~~in fact~~ tempting to speculate that the output of acetylcholine at high calcium concentrations is in fact on lowering the sodium concentration unaltered, so that if the quantal content could be determined directly at high outputs, the output/log calcium curves in low sodium concentrations would not be as predicted by the hypothesis (Fig. 4.2 p. 45 ) but as shown in Fig. (4.12). <sup>In order</sup> to retain that in a particular

Fig. (4.12)



**Fig. (4.12)** The hypothetical output/log calcium curves for normal NaCl and reduced NaCl based on the speculation that the output is unaltered by a reduction in the NaCl concentration at high calcium levels. The broken line is the output/log calcium curve that would then be expected in low sodium if no 'action potential effect' occurred.

low sodium chloride concentration 'the action potential effect' reduces the output by the same factor at all calcium concentrations, <sup>it would then be necessary to suppose that</sup> the relationship between the output and the log calcium concentration is not the same at all sodium concentrations but ~~would~~ increases in steepness as the sodium concentration was decreased (see Fig 4.12). Alternatively if a reduction in the sodium concentration does cause a parallel shift of the output/log calcium curve due to the interaction between calcium and sodium ions, the amplitude of the presynaptic action potential or the effect of the action potential on transmitter release must be a function of the calcium concentration.

It might be argued that at high calcium concentrations there is no interaction between calcium and sodium ions and that when the sodium chloride concentration is reduced there is no change in the action potential or that a change occurs in the relationship between the action potential and transmitter release which compensates for the reduction in the amplitude of the action potential. However, it has been shown that in the mammal (Gage and Quastel, 1965b; Elmqvist and Feldman, 1966) and in the frog (Birks and Burstyn, 1965, personal communication) at high calcium concentrations the replacement of sodium chloride by sucrose causes an acceleration of the potassium stimulated miniature e.p.p. frequency. This increase in the miniature e.p.p.



frequency probably reflects an increase in the probability of quantal release as a consequence of a reduction in the sodium chloride concentration.

The replacement of sodium chloride by sucrose or glycine could cause a parallel displacement of the output/log calcium curve by a less specific interaction between calcium and sodium ions in that a reduction in ionic strength would result in an increase in both the activity coefficient of calcium and in the number of receptors available to combine with calcium. Evidence in favour of this view is discussed in chapters 6 and 7.

SUMMARY

1) A hypothesis is outlined to explain the variation in the output of acetylcholine with changes in extracellular sodium concentration. It is proposed that reduction in the sodium ion concentration has two independent effects.

(a) If it is supposed that sodium and calcium ions compete for a 'receptor site' in the presynaptic nerve terminal and that only the calcium receptor complex is effective in allowing the release of acetylcholine, the output will tend to be increased by an increase in the proportion of receptor sites available to calcium ions and (b) by virtue of a reduction in the amplitude of the presynaptic action potential the output tends to be reduced.

2) Quantal contents of e.p.p.'s at various sodium chloride concentrations have been measured. At 0.23mM calcium, replacement of sodium chloride by sucrose or glycine leads to an increase in quantal content. At concentrations of calcium above 0.9mM, it is possible that little change in the quantal content occurs when sodium chloride is replaced by sucrose.

3) The results are compatible with the hypothesis outlined only if either (a) the interaction between sodium and calcium ions is such that a reduction in the sodium chloride concentration does not give rise to a parallel

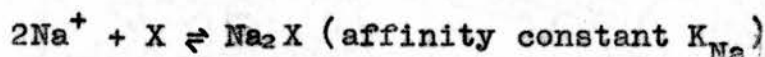
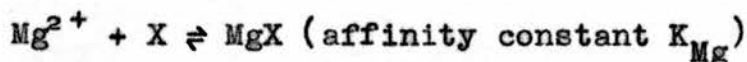
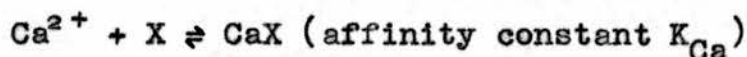
shift in the output of acetylcholine/log calcium relationship or (b) the amplitude of the action potential is a function of the calcium concentration at a constant sodium chloride concentration.

## **CHAPTER 5**

### **THE INTERACTION BETWEEN SODIUM, CALCIUM AND MAGNESIUM ON THE OUTPUT OF ACETYLCHOLINE**

In view of the uncertainty of the results in the previous chapter an alternative experimental approach was sought to the problem of whether sodium and calcium ions interact. Magnesium and calcium ions are known to interact and if their site of interaction in the nerve terminal was also the site at which sodium and calcium ions interact, this should be revealed by a study of the interaction of magnesium and calcium at different sodium concentrations.

Thus suppose that:



If  $\text{Ca}^{2+}$  is considered the agonist and  $\text{MgX}$  and  $\text{Na}_2\text{X}$  are taken to be inactive. Applying the law of mass action it is found that:-

$$\frac{\text{CaX}}{X_0} = \frac{K_{\text{Ca}} [\text{Ca}_1]}{1 + K_{\text{Ca}} [\text{Ca}_1] + K_{\text{Mg}} [\text{Mg}] + K_{\text{Na}} [\text{Na}]^2} \dots\dots(1)$$

where  $X_0$  is the total number of receptors.

In the absence of magnesium;

$$\frac{\text{CaX}}{X_0} = \frac{K_{\text{Ca}} [\text{Ca}_0]}{1 + K_{\text{Ca}} [\text{Ca}_0] + K_{\text{Na}} [\text{Na}]^2} \dots\dots\dots(2)$$

where  $[\text{Ca}_0]$  is the concentration of calcium in the absence of magnesium. On the assumption that a given amount of



transmitter is released when  $\frac{Ca \cdot X}{X_o}$  is constant then from (1) and (2):-

(c.f. Jenkinson, 1960 equ. 4)

$$\frac{[Ca_1]}{[Ca_0]} - 1 = \frac{K_{Mg} [Mg]}{1 + K_{Na} [Na]^2} \dots\dots\dots(3)$$

The expression can be rearranged:-

$$\frac{1}{A} = \frac{K_{Na} [Na]^2}{K_{Mg}} + \frac{1}{K_{Mg}} \dots\dots\dots(4)$$

$$\text{where } A = \frac{[Ca_1]}{[Ca_0]} - 1 \dots\dots\dots(4A)$$

Equation (4) has the form:-

$$y = b_1 x + c \dots\dots\dots(5)$$

$$\text{where } y = \frac{1}{A}, \quad b_1 = \frac{K_{Na}}{K_{Mg}}, \quad x = [Na]^2 \quad \text{and} \quad c = \frac{1}{K_{Mg}}$$

If therefore  $\frac{1}{A}$  is plotted against  $[Na]^2$ , a straight line should result with the slope  $K_{Na}/K_{Mg}$  and intercept on the 'y-axis' of  $\frac{1}{K_{Mg}}$ .

If a negligible number of receptor sites are left unoccupied when sodium, calcium and magnesium combine with the receptor as in the "ion exchange reaction", equations (1) to (5) from chapter 4 approximate to:-

$$\frac{[Ca_1]}{[Ca_0]} - 1 = \frac{K_{Mg} [Mg]}{K_{Na} [Na]^2} \dots\dots\dots(6)$$

which can be rearranged:-

$$\frac{1}{A} = \frac{K_{Na} [Na]^2}{K_{Mg}} \dots\dots\dots(7)$$

This is the equation of a straight line

$$y = bx \dots\dots\dots(8)$$

which runs through the origin.

If sodium, calcium and magnesium compete for the same receptor there should thus be a straight line relationship between  $y$ , the reciprocal of  $A$ , and the square of the sodium concentration (equations 5 and 8).

#### Experimental Procedure and Calculation of $[Ca_1]/[Ca_2]$

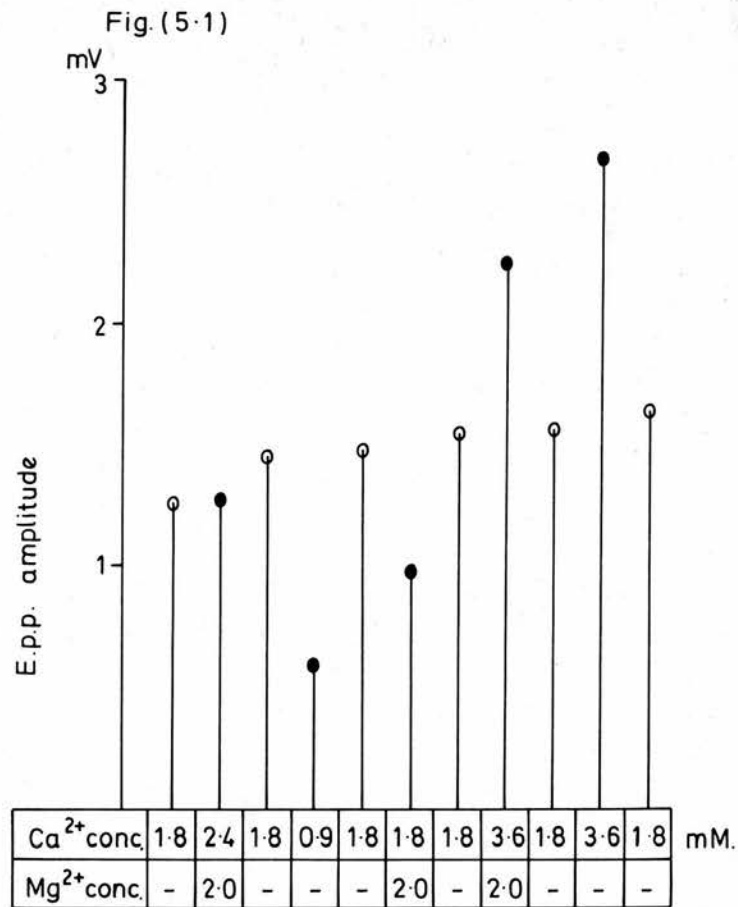
End-plate potentials were recorded either from single muscle fibres with intracellular electrodes or from the end-plate region of whole muscles by extracellular electrodes. (see chapter 2).

The amplitude of the e.p.p. was determined when the muscle was bathed alternately in a standard solution or in variants of this solution which contained the same sodium chloride and tubocurarine concentrations as the standard but different concentrations of calcium and magnesium. The standard and two of the variant solutions were magnesium free and contained three graded calcium concentrations selected so that the calcium concentration of the standard solution was in the middle of the range. The other three solutions all contained

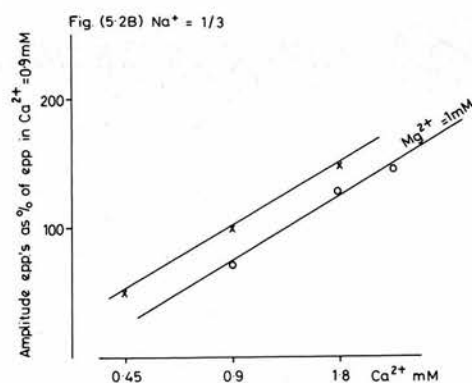
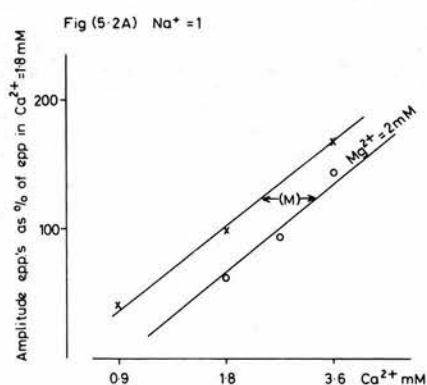
the same magnesium concentrations as well as a graded range of calcium concentrations whose mean was usually greater than that of the standard.

The mean amplitudes of the e.p.p.'s recorded in each of the variant solutions was expressed as a ratio of the mean of the amplitudes of the e.p.p.'s recorded in the standard solutions with which the variant was interchanged. Fig. (5.1 p. 92) shows the course of a typical experiment. The mean amplitudes of the e.p.p.'s recorded in the standard solution on six occasions remained fairly constant throughout the experiment whereas the mean amplitude in the variant solutions altered with the calcium and magnesium concentrations.

For each individual experiment, two output/log calcium curves were drawn which related mean amplitudes of the e.p.p.'s to the log of the calcium concentration in which they were recorded, in the presence and absence of magnesium. The output/log calcium curves were the two parallel regression lines which best fitted the six results. The horizontal distance between the two output/log calcium curves  $M$ , (Fig. 5.2 p. 93) was taken to be the log of  $[Ca_1]/[Ca_0]$  (equation 3). Although the output/log calcium lines can be fitted by eye,  $M$  was usually calculated from the data by a technique similar to that normally adopted for a 'six point assay', (Brownlee 1960). Table (5.1 p. 94) illustrates how  $M$  was calculated for the output/log calcium curves drawn in Fig. (5.2A).



**Fig. (5.1)** Time course of a typical experiment to show the effect of changing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentrations on the amplitude of the curarized e.p.p. The sodium concentration was 115.6 mM and tubocurarine concentration  $2.25 \times 10^{-6}$  M. Open circles denote amplitude of the e.p.p. in the standard solution  $\text{Ca}^{2+} = 1.8$  mM.



**Fig. (5.2A and B)** (A) Two output/log calcium curves in the presence and absence of 2 mM Mg when the NaCl concentration was 115.6 mM. (M) is the distance the two parallel regression lines are displaced (tubocurarine conc. =  $2.25 \times 10^{-6}$  M). (B) is a similar set of curves in 38.5 mM NaCl conc. (tubocurarine conc. =  $0.5 \times 10^{-6}$  M).



Table (5.1) The calculation of M, the ratio of  $[Ca_1]$ , to  $[Ca_0]$ , by the "six point assay" technique using the results illustrated in Fig. (5.2A). In Table (A) the amplitude of the e.p.p.'s in the three  $Ca^{2+}$  concentrations are tabulated for computing;  $x_0$  is the log of the  $Ca^{2+}$  concentration,  $y_0$  is the amplitude of the e.p.p.'s as a percentage of the amplitude in the standard solution  $Ca^{2+} = 18 \times 10^{-4}$  M. In Table (B)  $x_1$  and  $y_1$  are the corresponding results when the output/log calcium curve was determined in the presence of 2 mM  $Mg^{2+}$ .

TABLE A

No Magnesium

$1 \times 10^{-4}$ M $[Ca_0]$		9	18	36	Sum. $\Sigma$
log $[Ca_0]$ , $x_0$	$x_0$	0.9542	1.2553	1.5563	3.7658
	$x_0^2$				4.9083
E.p.p. amplitude, $y_0$	$y_0$	41	100	168	309
	$x_0 y_0$	39.1	125.5	261.5	426.1

TABLE B

2 mM Magnesium

$1 \times 10^{-4}$ M $[Ca_1]$		18	24	36	Sum. $\Sigma$
log $[Ca_1]$ , $x_1$	$x_1$	1.2553	1.3082	1.5563	4.1918
	$x_1^2$				5.9028
E.p.p. amplitude, $y_1$	$y_1$	65	94	144	303
	$x_1 y_1$	81.6	129.7	224.1	435.4

Mean regression coefficient  $b =$

$$\frac{\sum(x_0 y_0) - \frac{1}{3}(\sum x_0)(\sum y_0) + \sum(x_1 y_1) - \frac{1}{3}(\sum x_1)(\sum y_1)}{\sum(x_0)^2 - \frac{1}{3}(\sum x_0)^2 + \sum(x_1)^2 - \frac{1}{3}(\sum x_1)^2}$$

$$= 221.69$$

$$\begin{aligned} \frac{[Ca_0]}{[Ca_1]} = M &= \frac{\bar{y}_0 - \bar{y}_1}{b} + (\bar{x}_1 - \bar{x}_0) \\ &= \frac{\frac{1}{3}(309) - \frac{1}{3}(303)}{221.69} + \frac{\frac{1}{3}(4.1918) - \frac{1}{3}(3.7658)}{221.69} \end{aligned}$$

$$= 1.416$$

The experimental procedure was essentially the same whether the e.p.p.'s were recorded intracellularly from single muscle fibres or extracellularly from a whole muscle. The mean amplitude of the e.p.p.'s recorded extracellularly from a whole muscle, after it was bathed in the standard solution usually remained stable throughout an experiment. The preparation remained in good condition for 5 or 6 hours and was often used for a second experiment with a new series of solutions containing a different sodium and tubocurarine concentration.

The mean amplitude of e.p.p.'s recorded from single fibres of a muscle bathed in the standard solution were very much less constant and required the electrode insertion to remain stable for about 90 min. The mean amplitude of the e.p.p.'s in the standard solution was determined only three times during the experiment instead of before and after each variant solution in order to reduce the number of solution changes to eight. The majority of experiments at end-plates of single fibres were abandoned before all the solutions were tested because either the electrode was dislodged when the bath solution was changed or the amplitude of the e.p.p.'s recorded in the standard solution fell progressively through out the experiment inspite of an apparently stable resting potential.

The advantage of intracellular recording was that end-plates could be selected at which the presynaptic nerve remained excitable when the concentration of divalent ions increased. The extensor digiti IV muscle, which was used for the extracellular experiments, on the other hand, often developed nerve block in solutions with low sodium concentration when quite modest increases were made in the calcium and magnesium concentration.

## RESULTS

Fig. (5.2 p 93) shows the results of two experiments in which the e.p.p. was recorded extracellularly from the same muscle. In the first experiment, Fig. (5.2A) the sodium concentration was normal and the output/log calcium curves in the presence and absence of 2 mM magnesium were displaced by a distance (M) which was equivalent to a  $[Ca_1]$  to  $[Ca_0]$  ratio of 1.43. In the second experiment shown in Fig. (5.2B) in which the sodium concentration was only one-third of normal the distance between the two curves was the same, although the magnesium concentration was only half that in the previous experiment.

The value of A calculated for each of the two experiments from equation (4A)  $\left\{ A = \frac{[Ca_1] / [Ca_0] - 1}{[Mg]} \right\}$  was 208 in normal and 429 in one-third sodium. It is evident from the results of this experiment (1) and from four other experiments shown in table (5.2 p 98) that a decrease in the sodium concentration caused the value of A to increase.

Table (5.3) shows the average value of A obtained in 37 experiments. Seventeen of these experiments were made with intracellular recording.



Table (5.2) The results of five experiments in which A was determined in two different concentrations of sodium chloride on the same muscle using extra-cellular electrodes.

Expt. No.	Relative NaCl Concentration			
	1	5/6	2/3	1/3
	Value of A			
1	208	-	-	429
2	271	-	-	1794
3	-	245	308	-
4	-	249	298	-
5	-	185	200	-

TABLE (5.3)The average value of A in 37 experiments

	Relative sodium concentration				
	1	5/6	2/3	1/2	1/3
Value of A	242	253	266	700	1361
S.E.	15	27	22	68	232
No. of experiments	(11)	(6)	(6)	(7)	(7)

Competition between sodium, calcium and magnesium (according to equation (3) ) requires that there is a straight line relationship between the reciprocal of A, and the square of the sodium concentration , (equations 4 and 5). Fig. (5.3 p100) shows the plot of  $1/A$  against  $[Na]^2$ , and it seems clear that neither equations (4) nor (5) is obeyed. A more definitive test of equations 4 and 5 can be made by analysing the results of the individual experiments. In Fig. (5.4 p. 101) the 37 values of  $1/A$  have been plotted against  $[Na]^2$  and a regression line has been calculated as shown in table (5.4 p103).

The straight line is the calculated regression line  $y = 0.275x + 1.04$ . However, the deviations of the individual values of  $1/A$  from the regression line are not randomly distributed and there is a systematic deviation from linearity. The regression function can be shown to

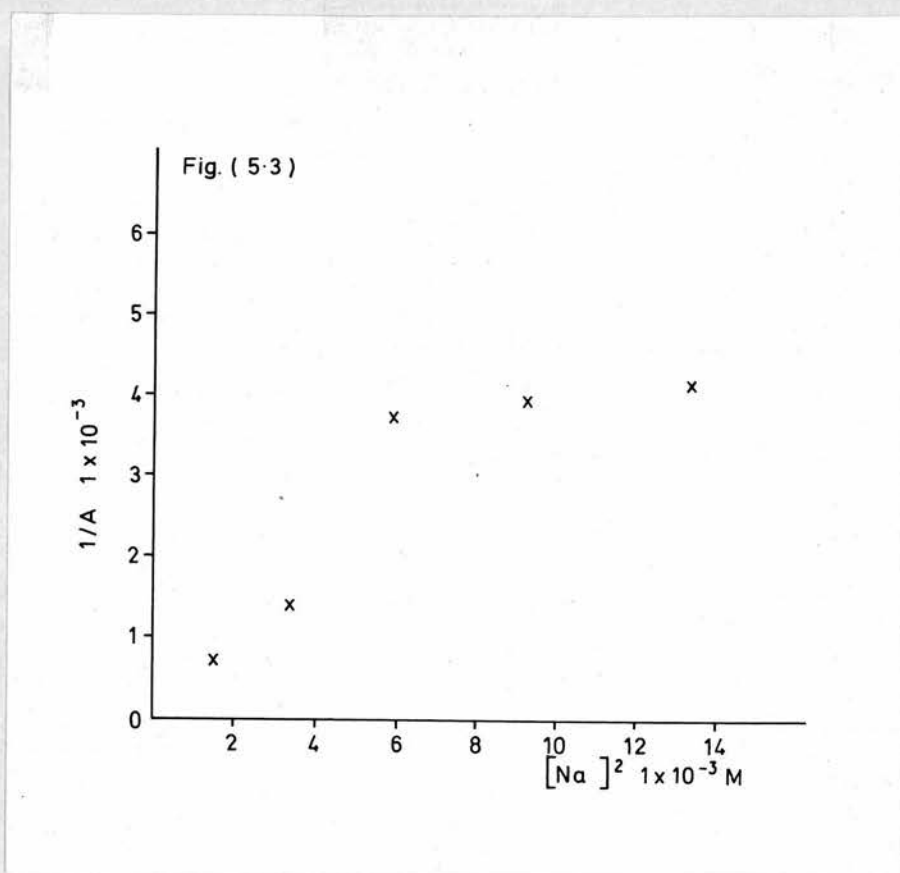
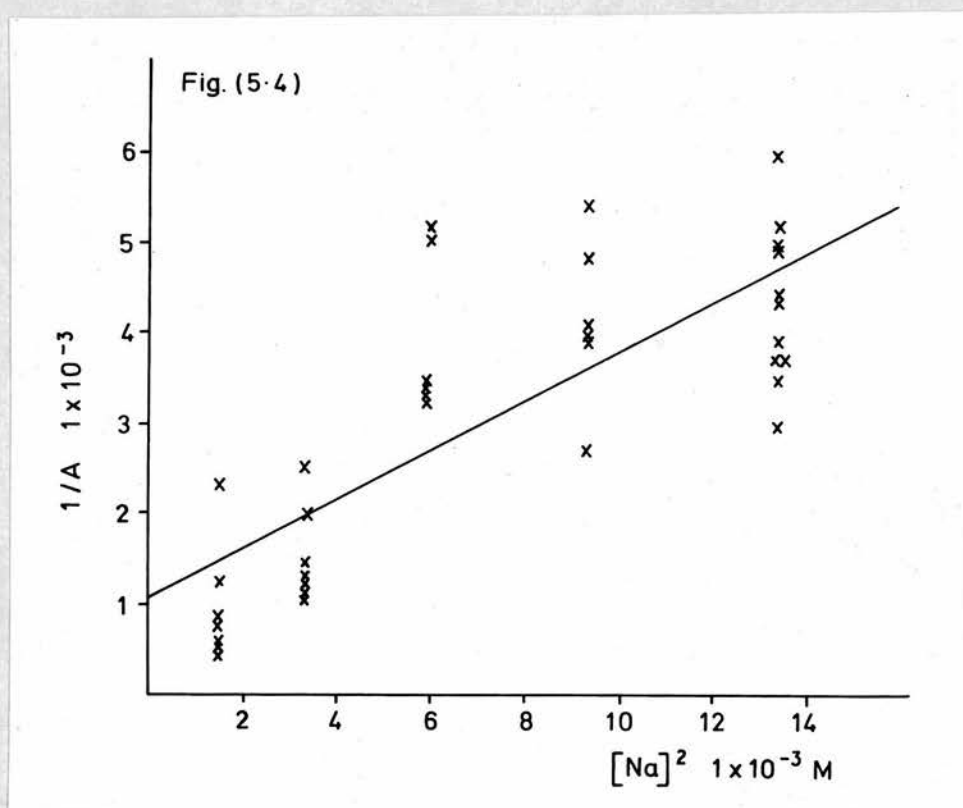


Fig. (5.3) The relationship between the reciprocal of the average values of A in Table (5.3) plotted against  $[Na]^2$ .



**Fig. (5.4)** The 37 values of  $1/A$  plotted against  $[Na]^2$ . The line is the calculated regression line  $y = 0.275x + 1.04$  calculated as shown in Table (5.4).

depart from linearity by the analysis of variance (Table 5.5 p104 ). The ratio of the variance of the column means about the regression line to the variance within columns is much greater than the critical value for F for the appropriate degrees of freedom ( $P = 0.05$ ).



Table (5.4) Calculation of the regression line  
 $y = 0.275x + 1.04$  from the 37 values of  $1/A$  plotted  
 against  $[Na]^2$ . (See Fig. (5.4) ).

Relative NaCl conc.	1	5/6	2/3	1/3	1/3
$[Na]^2$ ( $1 \times 10^{-3}$ )	13.36	9.28	5.94	3.34	1.48
Values of $1/A$ ( $1 \times 10^{-3}$ )	3.45	4.08	3.25	1.15	0.54
	2.94	4.02	3.36	1.21	2.33
	4.81	2.68	5.15	1.45	0.56
	3.69	5.41	3.42	2.48	1.26
	3.86	3.88	3.28	1.19	0.47
	3.73	4.83	5.05	1.30	0.76
	5.18			1.99	0.84
	5.95				
	4.93				
	4.39				
	4.37				
Column Means	4.30	4.15	3.92	1.54	0.97

Overall mean of values  $1/A$  = 3.06

Mean of the variable  $[Na]^2$  = 7.35

Sum of squares of deviations of  $[Na]^2$  from mean  $[Na]^2$   
 = 785.4

Sum of products = 216.1

Regression coefficient =  $\frac{216.1}{785.4}$  = 0.275

$$y = bx + c$$

$$= 0.275 (x - 7.35) + 3.06$$

$$y = 0.275x + 1.04$$

Table (5.5) Analysis of Variance of the 37 values of  $1/A$  at different  $[Na]^2$  from the regression line  $y = 0.275x + 1.04$  (see Table (5.4) and Fig. (5.4) ).

Analysis of Variance	Sum of Squares	Degrees of Freedom	Variance	F	Critical Value of F P=0.01
1) for variation within columns	20.28	32	0.633	8.37	4.46
2) of the deviation of the column means from the regression line	15.91	3	5.303		
3) of the deviation of the regression line from the overall mean	59.46	1	59.459		
Total deviation of the individual values from the overall mean	95.72	36			

## DISCUSSION

The non-linear nature of the relationship between  $1/A$  and the square of the sodium concentration does not support the idea that sodium, calcium and magnesium ions compete for the same receptor site, at least in the way outlined in the simple hypothesis at the beginning of the chapter.

One way in which the hypothesis could be modified is to assume the existence of the species  $NaX$  as well as  $CaX$ ,  $MgX$  and  $Na_2X$ . According to this scheme

$$A = \frac{K_{Mg}}{1 + K_{Na'} [Na] + K_{Na} [Na]^2} \dots\dots\dots (9)$$

where  $K_{Na'}$  is the affinity constant for  $NaX$ . No values can be chosen for  $K_{Mg}$ ,  $K_{Na}$  and  $K_{Na'}$  however, which give rise to a relationship between  $1/A$  and  $[Na]^2$  similar to the observed relationship.

If it is assumed that no specific interaction between sodium and the divalent cations occurs, the results may be used to calculate  $K_{Mg}$  on the basis that calcium and magnesium ions alone compete for a single site (see Jenkinson, 1957). In fact  $K_{Mg}$  is given by the values of  $A$  shown in Table (5.3 p 99). In normal sodium Ringer the value of  $K_{Mg}$  was 242 which agrees with the value of  $(\frac{1}{4 \times 10^{-3} M} = 250)$  obtained by Jenkinson (1957).

A possible explanation for the increase in  $K_{Mg}$  when the sodium chloride concentration is reduced but is that this reflects a change in the ionic strength. For example, if the receptor site was in the form of an anion  $X^{2-}$  the true affinity constant would be given by

$$K_{Mg}^* = \frac{\gamma_{MgX} [MgX]}{\gamma_{Mg} [Mg] \gamma_X [X]}$$

The observed affinity constant is say

$$K_{Mg} = \frac{[MgX]}{[Mg] [X]}$$

$$\therefore K_{Mg} = \frac{K_{Mg}^* \gamma_{Mg} \gamma_X}{\gamma_{MgX}}$$

where  $\gamma$  denotes activity coefficient on a molal basis.

If  $[MgX]$  is unionized its activity coefficient will not vary appreciably with ionic strength.  $\gamma_{Mg}$  and  $\gamma_X$  however, will increase with <sup>a decrease in</sup> ionic strength and hence the observed affinity constant will also increase.

If the somewhat speculative values of  $\gamma$  which can be calculated from the Debye - Hückel equation (e.g. Long, 1961) are applicable then  $K_{Mg}$  would increase by factors of 1.12, 1.24, 1.50 and 1.82 when the sodium chloride concentration was reduced to 5/6, 2/3, 1/2 and 1/3 respectively. The observed values of  $K_{Mg}$ , however, were

increased in the appropriate sodium concentrations by factors of 1.05, 1.10, 2.9 and 5.6.

If the increase in  $K_{Mg}$  is due to a decrease in ionic strength when sodium chloride is replaced by sucrose, an experimental test of this idea would be to determine  $K_{Mg}$  when sodium chloride is replaced by an ionized substitute.



## SUMMARY

1) The interaction between sodium and calcium ions is re-examined on the assumption that the interaction between calcium and magnesium ions will alter as the sodium concentration is reduced, if sodium, calcium and magnesium ions interact at the same site in the nerve terminal.

2) The values of  $K_{Mg}$  (the affinity constant of magnesium for receptor sites at which calcium and magnesium interact) were determined at five different sodium chloride concentrations. The values were as follows:  
in normal sodium chloride  $K_{Mg} = 242 \pm 15$  (S.E.),  
in five-sixth normal  $253 \pm 27$ , in two thirds normal  $266 \pm 22$ , in one-half normal  $700 \pm 68$  and in one-third normal sodium chloride  $1361 \pm 232$ .

3) The relationship between the experimental results and the square of the sodium concentration was not as predicted by the hypothesis that sodium, calcium and magnesium ions all interact at the same site.

## CHAPTER 6

THE REPLACEMENT OF SODIUM CHLORIDE BY SUBSTITUTES  
WHICH MAINTAIN THE IONIC STRENGTH BUT DO  
NOT SUSTAIN THE AMPLITUDE OF THE  
PRESYNAPTIC ACTION POTENTIAL

The effect of ionized sodium substitutes on the quantal content of the e.p.p.

When sodium chloride is replaced by sucrose there is an unavoidable lowering of the ionic strength. This will be expected to have at least two effects. The activity coefficient of the extracellular calcium will be increased and the number of available strategic sites with which calcium is presumed to combine will also increase. At a particular sodium chloride concentration (i.e. at constant ionic strength) these effects might be supposed to be independent of the external calcium concentration; the hypothesis outlined in chapter 4 should not therefore, lead to predictions which are essentially different from those which result from the idea that there is competition between sodium and calcium ions. Disregarding the effect on the action potential, the log calcium - quantal content curve should be displaced to the left by a constant amount even if the effect on the quantal content was due to the change in ionic strength rather than competition. However, too little is known of the nature of the strategic sites to exclude the possibility of other complicating effects of changes in ionic strength; the results of chapter 4 do not therefore rule out the 'ionic strength' hypothesis. It is in fact, difficult to envisage a satisfactory direct test of this hypothesis but a minimum requirement which should be satisfied is that 'inert' ionic substitutes for sodium chloride should

lead to a reduction in quantal content of the e.p.p. at all calcium concentrations. The results that would be expected on the hypothesis that sodium and calcium are competitive have already been outlined in chapter 4.

The sodium chloride substitutes studied were methylammonium chloride ( $\text{CH}_3\text{NH}_3\text{Cl}$ ), ethylammonium chloride ( $\text{CH}_3\text{CH}_2\text{NH}_3\text{Cl}$ ) and tris chloride (tris-(hydroxymethyl)-aminomethane). Deck (1958) has classified methylammonium chloride and ethylammonium chloride as being inert as far as the process which generates the nerve axon spike is concerned. The replacement of sodium chloride by methylammonium and ethylammonium chlorides should cause a reduction in the presynaptic action potential and hence (on the 'ionic-strength' hypothesis) a marked reduction in the amount of transmitter released. The effect of replacement of sodium by alkyl-derivatives of ammonium on the end-plate potential has been studied by Furukawa and Furukawa (1959) at the frog neuromuscular junction, but they did not determine quantal contents.

Tris chloride has been used as an inert substitute for sodium chloride by Lüttgau and Niedergierke (1957, 1958) on frog heart muscle and by Fatt and Ginsborg (1958) on crustacean muscle.

## Solutions

In normal Ringer 1.75 ml of an isotonic phosphate buffer solution containing 63 mM  $\text{Na}_2\text{HPO}_4$  and 23 mM of  $\text{NaH}_2\text{PO}_4$  per litre was sufficient to maintain the pH between 6.5 and 6.8. When the sodium chloride was replaced by methylammonium and ethylammonium chlorides the solution became distinctly acid unless larger amounts of buffer were added. The volume of buffer required to retain the pH within the range 6.5 to 6.8 never exceeded 10 ml and the same volume was also added to the control solutions. No additional buffer was required when the sodium chloride replacement was tris chloride since isotonic tris chloride was prepared by titrating tris-(hydroxymethyl)-aminomethane with hydrochloric acid until the desired pH was attained. At a pH of 6.8 all the methylammonium chloride will be in the form of  $\text{CH}_3\text{NH}_3^+$  and  $\text{Cl}^-$  as the pK is 10.62. Similarly ethylammonium chloride will be present as  $\text{CH}_3\text{CH}_2\text{NH}_3^+$  and  $\text{Cl}^-$ . One litre of isotonic tris chloride solution was prepared by the addition of 118 mM of HCl to 122 mM of tris-(hydroxymethyl)-aminomethane  $\text{C}(\text{CH}_2\text{OH})_3\text{NH}_2$ . Since the pK of  $\text{C}(\text{CH}_2\text{OH})_3\text{NH}_2$  is 8.22, one litre of the Ringer solution at a pH of 6.8 will contain 118 mM of  $\text{C}(\text{CH}_2\text{OH})_3\text{NH}_3^+$  and  $\text{Cl}^-$  and 4 mM of  $\text{C}(\text{CH}_2\text{OH})_3\text{NH}_2$ .



## RESULTS

The results to be described in this section are concerned with the changes in quantal content which occurred within a short time (5 - 10 min) after replacing sodium chloride in the bathing fluid. On prolonged exposure to solutions containing either methyl- or ethylammonium chlorides further changes occur which are described in a later section.

In low Calcium. As described in chapter 2 intracellular records were made of evoked and spontaneous e.p.p.'s from frog sartorii muscles. They were bathed in solutions which contained the same low calcium concentration (0.3 mM) but different sodium concentrations prepared by the replacement of sodium chloride with an isotonic solution of either methylammonium, ethylammonium or tris chlorides. The microelectrode was inserted into the end-plate region of a muscle fibre and the nerve was stimulated at 3.2 second intervals. One hundred or more evoked e.p.p.'s and as many spontaneous e.p.p.'s as possible were recorded over a 5 minute period. After a record was obtained in the first sodium concentration the microelectrode was left undisturbed while the solution in the bath was changed and allowed to equilibrate for a period of 5 minutes. A further series of evoked and spontaneous e.p.p.'s were then recorded in the new solution.

Methylammonium Chloride. When one half or one third of the sodium chloride in normal Ringer was replaced by methylammonium chloride there was a substantial decrease in the mean amplitude of the evoked e.p.p. A small proportion of this decrease may be attributed to a decrease in the sensitivity of the post-synaptic membrane to acetylcholine as the mean amplitude of the spontaneous end-plate potentials decreased slightly. The major part of the decrease in amplitude, however, represented a considerable fall in the number of quanta released per nerve impulse. The decrease in the mean amplitude of the evoked end-plate potential was accompanied by an increase in the proportion of stimuli which failed to elicit a response. The mean number of quanta per nerve impulse predicted from the proportion of "failures" to trials agreed with the two estimates calculated from the mean amplitudes of the spontaneous and evoked end-plate potentials and from the variance of the amplitudes of the evoked end-plate potentials. When the reverse change was made and the methylammonium chloride replaced by sodium chloride the mean amplitude of the evoked end-plate potentials increased in size and the number of "failures" decreased to zero.

Table (6.1) shows that in five experiments the average quantal content of the e.p.p.'s recorded in the solution containing equal parts of sodium and methylammonium chlorides was 2.5 times less than in normal sodium. In

TABLE (6.1)

The effect of replacement of sodium chloride by methylammonium chloride on the quantal content.

Exp. No.	Soln.	Amplitude of		Quantal Content			
	Na	EPP (mv)	Mepp	m <sub>1</sub>	m <sub>0</sub>	m <sub>2</sub>	m <sub>3</sub>
1	1	6.52	0.94	7.0		13.1	8.6
	1/2	0.86	0.39	2.2	2.4	1.9	
2	1	6.58	0.49	13.5		16.9	14.6
	1/2	0.49	0.24	2.0	2.0	2.2	2.1
3	1	0.76	0.41	1.8	2.4	2.0	1.9
	1/2	0.21	0.37	0.6	0.7	0.6	0.6
4	1	3.9	0.47	8.3			
	1/3	0.81	0.32	2.5	2.3		
	1	3.6	0.42	8.6			
5	1/2	1.8	0.32	5.7		5.6	5.7
	1	1.93	0.32	6.2		6.5	6.2
6	1/2	4.63	0.69	6.7		9.6	7.6
	1	7.40	0.54	13.9		14.7	14.2

The calcium concentration was 0.3 mM in all solutions.

one experiment when two thirds of the sodium chloride was replaced by methylammonium chloride the quantal content was reduced by a factor of 3.4.

Ethylammonium Chloride. In two experiments in which the sodium substitute was ethylammonium the quantal content increased from 0.4 quanta in one third sodium to 6.7 in normal sodium and from 0.83 in one half sodium to 8.8 in normal sodium. In solutions in which one half of the sodium chloride was replaced by ethylammonium chloride, the amplitudes of the miniature e.p.p.'s were greatly reduced and they became lost in the base line noise.

Tris Chloride. Even small replacements of sodium chloride by tris chloride reduced the amplitudes of the miniature e.p.p.'s to a level at which they could not be distinguished from the base line noise. In one experiment when only one quarter of the sodium chloride was replaced by tris chloride the mean amplitude of the evoked e.p.p.'s was reduced from 4.2 mV to 0.88. This change represented a two fold decrease in the quantal content from 12 to 6 quanta and a 2.4 fold reduction in the unit size. In an other experiment the quantal content increased from 0.53 quanta in one third sodium to 3.7 in nine tenths of the normal sodium.

If the reduction in quantal content which occurs when sodium chloride is replaced by either methylammonium,

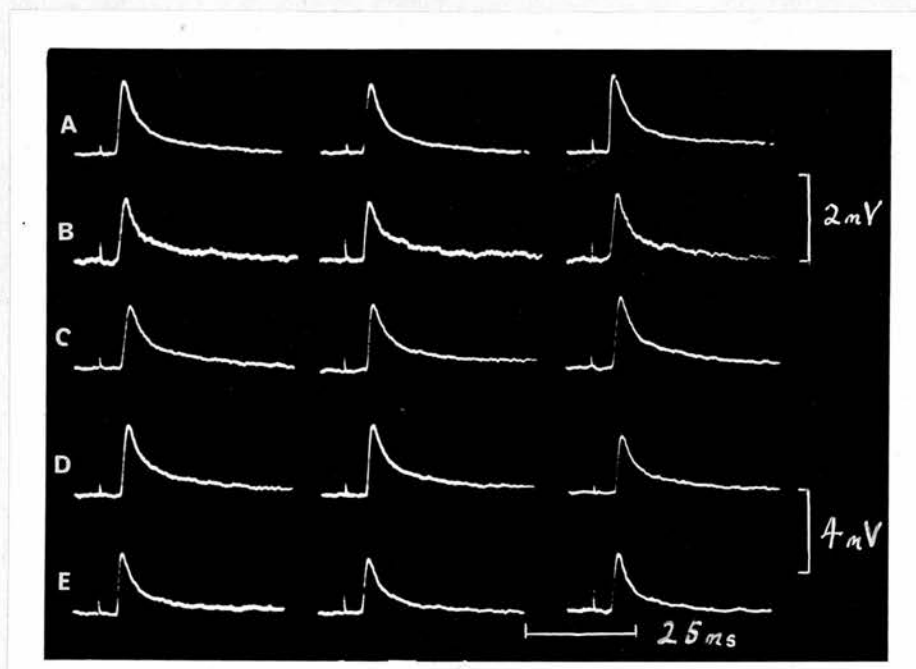
ethylammonium or tris chlorides due to a reduction in the amplitude of the presynaptic action potential rather than the result of competitive inhibition, the quantal content would be expected to be the same in a particular sodium concentration regardless of the nature of the ionized substitute. In two experiments in which the calcium concentration was one quarter of the normal, the quantal content calculated from the variance of the amplitudes of the e.p.p.'s (equation 4, chapter 3) was unaltered when a solution containing one half of the sodium chloride replaced by ethylammonium chloride was changed for another in which the sodium substitute was tris chloride. In both experiments the mean amplitude of the evoked e.p.p.'s was reduced 2.2 fold when the ethylammonium chloride was replaced by tris chloride.

In summary, methylammonium, ethylammonium and tris chlorides all cause a reduction in the amount of transmitter released per nerve impulse, when they replace the sodium chloride from low calcium Ringer. Ethylammonium and tris chlorides caused equal reductions in the quantal content. In methylammonium chloride the amplitudes of the spontaneous end-plate potentials were reduced very little but in ethylammonium and tris chlorides they were depressed to a greater degree presumably due to a reduction in the sensitivity of the post-synaptic membrane to the transmitter.



Normal Calcium. In two experiments the mean amplitudes of evoked end-plate potentials recorded by intracellular electrodes from the end-plate region of a curarized muscle bathed in normal calcium Ringer were reduced by slightly more than 50% when one half the sodium was replaced by methylammonium chloride. During the next 20 minutes, however, the amplitudes of subsequent evoked end-plate potentials gradually increased in size until their mean amplitude was two and three times greater than immediately after the solution change. On return to normal Ringer the mean amplitude of the evoked end-plate potential was smaller than that of the last evoked end-plate potentials recorded in the presence of methylammonium. (Fig. 6.1). If it is assumed that a replacement of sodium by methylammonium chloride causes little alteration in the post-synaptic sensitivity to acetylcholine (see p.113) and that there is no interaction between tubocurarine and methylammonium chloride, the reduction in the mean amplitude of the e.p.p.'s immediately after the replacement of sodium chloride by methylammonium chloride represents a 2 - 3 fold reduction in the output of transmitter.

The reduction in the output of transmitter in normal calcium together with the qualitatively similar results in lower calcium are consistent with the idea that the reduction is due to a decrease in the amplitude of the presynaptic potential. To establish this claim more



**Fig. (6.1)** The effect of replacing one-half of the NaCl by methylammonium chloride on the e.p.p. amplitude. Both solutions contained 1.8 mM  $\text{Ca}^{2+}$  and  $7 \times 10^{-6}$  M tubocurarine. A - E.p.p.'s recorded in normal NaCl solution (mean amplitude 3.62 mV). B - E.p.p.'s recorded from the same end-plate 3 min after one-half of the NaCl was replaced by methylammonium chloride (mean amplitude 1.41 mV). Note 2x increase in gain. C - E.p.p.'s recorded 15 min after the replacement (mean amplitude 2.58 mV). D - E.p.p.'s recorded 27 min after the replacement (mean amplitude 3.47 mV). F. - E.p.p.'s after the return to full NaCl (mean amplitude 2.18 mV).

fully it would be necessary to show that the size of the decrease is independent of (a) the calcium concentration and (b) the actual chemical structure of the ionized substitute. The large variability of the results in low calcium suggest that a very large number of experiments would be required. At higher calcium the difficulties involved in interpreting the amplitude of curarized e.p.p.'s in terms of quantal content may lead to an ambiguous result. In any event the effects of prolonged exposure to methylammonium chloride on the quantal content described later would detract from the value of these results.

Progressive changes in quantal content during  
prolonged exposure to methylammonium chloride.

In experiments described earlier, when the bathing solution contained the normal calcium concentration, the mean amplitudes of e.p.p.'s recorded from curarized muscle, was found to increase progressively 2 - 3 times in the 20 mins following the replacement of sodium chloride. The amplitude of the e.p.p. recorded externally from a curarized muscle continued to increase for periods of at least 40 mins (after the initial decline in amplitude) when sodium chloride was replaced by ethylammonium chloride. In order to establish whether this effect was due to a presynaptic action of methylammonium which caused a progressive increase in the output of transmitter or due to a post-synaptic action

whereby the sensitivity of the post-synaptic membrane to acetylcholine increased, the action of methylammonium chloride on the quantal content and on the miniature e.p.p. size was re-examined at low calcium in the absence of tubocurarine.

Table (6.2 p.121) shows that the replacement of one-half of the sodium chloride in low calcium Ringer solution by methylammonium chloride caused an immediate reduction of the quantal content from 8.6 to 2.3 quanta and from 14.6 to 2.1 quanta in two individual experiments. More prolonged exposure to methylammonium chloride, however, led to an increase from 2.3 to 10.1 quanta 30 min later in the first experiment and from 2.1 to 6.4, 40 min later in the other.

Prolonged exposure to methylammonium chloride appears to have a presynaptic action which leads to a progressive increase in the quantal content of the e.p.p.

The effect of sodium substitutes on the potassium-stimulated miniature e.p.p. frequency.

In the introduction, (p. 12) it was argued that in the mammal and the frog, the probability of release of an individual quantum is reflected by the potassium stimulated miniature e.p.p. recurrence frequency.

In the mammal, Gage and Quastel (1965b) have shown that the replacement of 50 - 70% of the sodium chloride

Table (6.2) The results of two experiments which show the immediate and more prolonged effects of replacing sodium chloride by methylammonium chloride. Quantal contents estimated as weighted mean,  $m_3$ , (see chapter 3 equations 2, 4 and 9).

Experiment Number	$Na^+=1$	$Na^+=1/2$ and the replacement is $CH_3NH_3Cl$			
1.(min after replacement)	-	(5-10)	(10-15)	(20-25)	(33-38)
E.p.p. amplitude	6.52	0.86	1.29	2.97	4.00
M.e.p.p. amplitude	0.94	0.39	0.44	0.55	0.45
Quantal content	8.6	2.3	3.2	5.6	10.1
2.(min after replacement)		(5-10)	(10-15)	(23-28)	
E.p.p. amplitude	6.58	0.98	3.22	3.58	
M.e.p.p. amplitude	0.49	0.48	0.58	0.48	
Quantal content	14.6	2.1	6.1	6.4	



in potassium rich Ringer solution by sucrose leads to a striking increase in the miniature e.p.p. recurrence frequency. In the frog, the potassium stimulated spontaneous e.p.p. discharge was found by Birks and Burstyn (1965) to be increased when part of the extracellular sodium chloride was replaced by sucrose. Since the probability of release of the individual quantum is known to increase in this situation, the results of Gage and Quastel and Birks and Burstyn strongly suggest that the extent of the potassium stimulated miniature e.p.p. frequency does reflect an increase in probability of release. If this increase in probability was due to the withdrawal of sodium (decrease in competitive inhibition) rather than a reduction in the ionic strength, the potassium stimulated miniature e.p.p. discharge would be expected to increase when the sodium chloride is replaced by the ionized substitutes. If this were the case, the contrast between the decrease in the quantal content which occurs when sodium chloride is replaced by the ionized substitutes and the increase which occurs when the replacement is sucrose would be attributed (on the basis of the hypothesis outlined in chapter 4) to a greater reduction in the amplitude of the presynaptic action potential when the sodium chloride replacement is an ionized substitute instead of sucrose.

The experiments described below, however, show that the replacement of sodium chloride by methylammonium and

ethylammonium chlorides does not lead to an increase in the potassium stimulated miniature e.p.p. frequency. The increase in the quantal content caused by the replacement of sodium chloride by sucrose rather than methylammonium or ethylammonium chlorides may, therefore, be due to an increase in the probability of release caused by a reduction in the ionic strength. On this basis, it would be unnecessary to postulate that the amplitude of the presynaptic action potential in low extracellular sodium concentration is sensitive to the nature of the replacement.

#### Procedure.

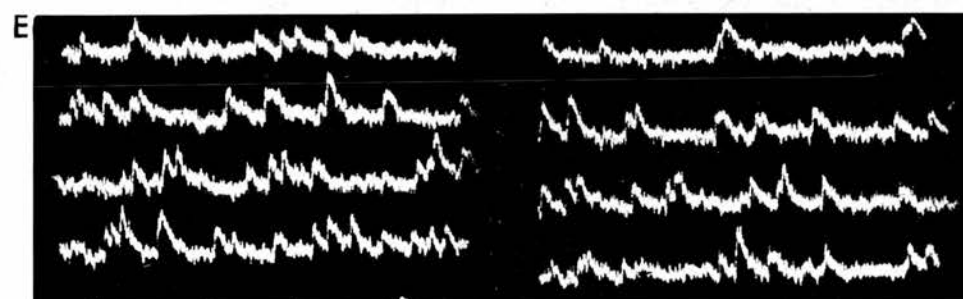
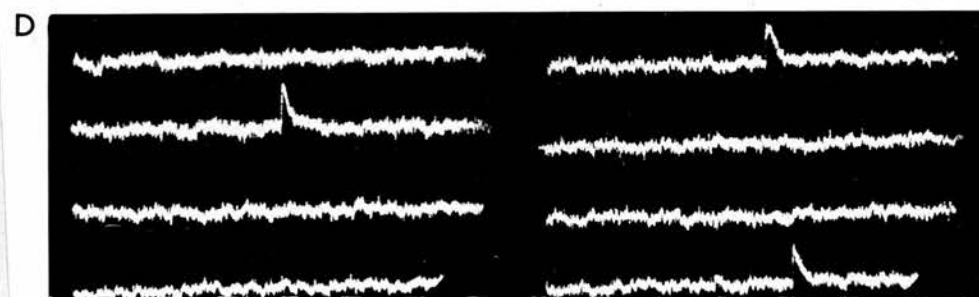
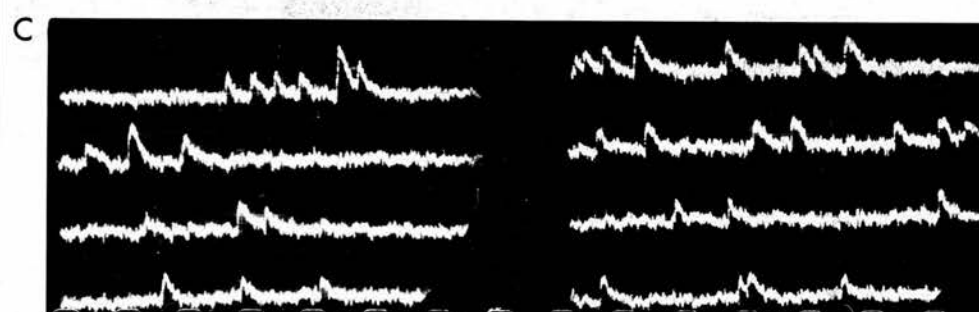
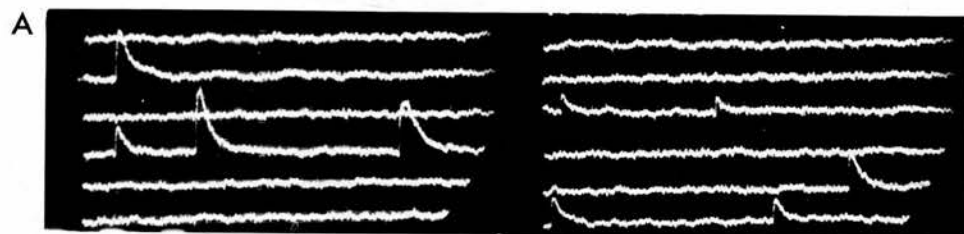
Miniature e.p.p.'s were recorded at intervals with a microelectrode which was maintained inserted in the same end-plate of a muscle fibre, the muscle being bathed in the different solutions. Each solution was allowed to equilibrate with the muscle for four minutes before a new series of miniature e.p.p.'s were recorded. The end-plates were usually located with the muscle bathed in normal Ringer solution. The subsequent solutions were prepared by mixing one volume of a solution which contained the normal sodium chloride concentration, twice the desired calcium concentration (i.e.  $2 \times 0.225$  or  $2 \times 1.8$  mM) and twice the required neostigmine concentration (i.e.  $2 \times 10^{-6}$  w/v) with an equal volume of either sodium, methylammonium, ethylammonium chlorides or sucrose. The miniature e.p.p. frequency was

determined from photographic records of oscilloscope sweeps which were approximately 0.5 sec in duration and were repeated at 2 sec intervals for a period of 5 min or more.

## RESULTS

Fig. (6.2) illustrates the records from which the miniature e.p.p. recurrence frequencies were calculated, in four different solutions during a typical experiment. In one eighth of the normal calcium Ringer the miniature e.p.p. recurrence frequency was 1.1/sec and an increase in the potassium concentration from 2 mM to 9.5 mM increased the frequency to 1.9/sec. When the solution was replaced by a solution in which one-half of the sodium chloride had been replaced by sucrose the miniature e.p.p. recurrence frequency rose almost four fold to 7.3/sec. The miniature e.p.p. recurrence frequency declined rapidly to 0.6/sec which is less than the original level in normal sodium, when the one-half sucrose solution was exchanged for a solution in which the sodium substitute was methylammonium chloride. A further exposure to the solution in which the sodium replacement was sucrose caused an even greater increase in the miniature e.p.p. frequency to 17.6/sec.

The results of 8 similar experiments in which the potassium stimulated miniature e.p.p. recurrence frequency in solutions containing either normal sodium



400 ms

or one half of the sodium replaced by either sucrose, methylammonium or ethylammonium was compared, are shown in Table (6.3).

The miniature e.p.p. recurrence frequency in normal sodium was increased on average 1.4 fold when the potassium concentration was raised from 2.0 to 9.5 mM. When one-half of the sodium chloride was replaced by sucrose the miniature e.p.p. recurrence frequency was on average 5 times greater than in either full sodium or when one-half of the sodium was replaced with methylammonium or ethylammonium chlorides.

The experiments confirm the finding of Gage and Quastel (1965b) and Birks and Burstyn (1965) that replacement of sodium chloride by sucrose leads to an increase in the potassium stimulated miniature e.p.p. recurrence frequency. In contrast, the potassium stimulated miniature e.p.p. recurrence frequency is restored to about the frequency in normal sodium when the sucrose is exchanged for either methylammonium or ethylammonium chlorides. Replacement of sodium chloride by methylammonium chloride or ethylammonium chloride does not appear to affect the probability of release.



Table (6.3) The results of 8 experiments to compare the effects of replacement of sodium chloride by either sucrose or methyl- or ethyl- ammonium chloride on the potassium stimulated (9.5 mM) miniature e.p.p. discharge frequency.

		Miniature e.p.p. discharge frequency/sec.					
Exp. No.	Ca <sup>2+</sup> mM	NaCl	NaCl + KCl	$\frac{1}{2}$ NaCl + KCl + sucrose	$\frac{1}{2}$ NaCl + KCl + CH <sub>3</sub> NH <sub>3</sub> Cl	$\frac{1}{2}$ NaCl + KCl + CH <sub>3</sub> CH <sub>2</sub> NH <sub>3</sub> Cl	$\frac{1}{2}$ NaCl + KCl + sucrose
1	0.23	1.1	1.9	7.3	0.6	-	17.6
2	1.8	0.6	2.6	12.7	1.4	-	-
3	1.8	0.4	0.9	33.4	0.9	-	-
4	0.23	0.7	1.3	2.7	-	0.9	11.0
5	1.8	3.9	4.4	12.5	-	5.9	-
6	1.8	3.6	3.5	7.2	-	3.7	-
7	1.8	1.5	-	11.8	-	1.7	34.6
8	1.8	-	-	10.8	-	4.5	14.2

## DISCUSSION

The most interesting result is that the replacement of a proportion of the sodium chloride from Ringer solution by one of the ionized substitutes, methylammonium, ethylammonium or tris chlorides, gives rise to a decrease in the output of transmitter. If this decrease is a reflection of the reduction in the presynaptic action potential, the decrease in the quantal content should be related to the reduction in the sodium chloride concentration and of the same magnitude at all calcium concentrations.

In low calcium concentrations the decrease in quantal content caused by a reduction in the sodium chloride concentration to one-half was extremely variable; the large number of experiments that would be necessary to establish the size of this reduction with precision, could not unfortunately be made in the available time. In five experiments the average decrease in quantal content was by a factor of 2.5 (range 1.1 to 6.7). This is of the same order of magnitude although smaller than the factor of 6.4 which would be expected on the basis of Liley's (1956c) hypothesis (see p. 17).

In higher calcium concentrations it would be much more difficult to establish the magnitude of the decrease in the quantal content caused by a particular reduction in sodium concentration. Changes in the amplitude of

the curarized e.p.p. when sodium chloride is replaced by a substitute may be due to changes in sensitivity of the post-synaptic membrane to acetylcholine or tubocurarine. When the substitute for sodium chloride can also cause a progressive increase in the quantal content as described above, an ambiguous result seems unavoidable.

If the ionized substitutes are truly inert and the decrease in quantal content a consequence of the reduction in the presynaptic action potential, the quantal content should be unaltered by differences in the chemical structure of the ionized substitutes for sodium chloride. In two experiments it was shown that the quantal content was unaltered when tris chloride was exchanged for ethylammonium chloride. It is, however, evident that a more exhaustive investigation of the ionized substitutes for sodium chloride may afford an opportunity to examine the effect of calcium for example, on the relationship between the quantal content and the amplitude of the presynaptic action potential. However, interpretation of these experiments is at present ambiguous since as is described in chapter 7 the replacement of sodium chloride by substances which maintain the ionic strength but should not lead to a reduction the presynaptic action potential have also been found to cause a reduction in the quantal content.

In the introduction it was mentioned that Furukawa and Furukawa (1959) had examined the effects of replacing sodium chloride with alkyl-derivatives of ammonium on the amplitude of the e.p.p. In contrast, to the results presented above Furukawa and Furukawa found that the amplitude of the evoked e.p.p. recorded from curarized muscle by an intracellular microelectrode, was increased rather than reduced when four-fifths of the sodium chloride in their Ringer solution was replaced by either methylammonium or ethylammonium chlorides. In the uncurarized muscle Furukawa and Furukawa (1959) found that the amplitude of the e.p.p. recorded from a muscle bathed by Ringer solution from which four-fifths of the sodium chloride had been replaced by sucrose, became larger and gave rise to a feeble action potential when the sucrose was replaced by methylammonium or ethylammonium chlorides.

If the amplitude of the e.p.p. was a measure of the output of transmitter these two experiments conflict with the results described above in that they suggest that the output of transmitter is increased or unaltered when the sodium chloride is replaced by an ionized substitute and that the output is also increased when the sucrose replacing sodium chloride is exchanged for an ionized substitute. There is no obvious explanation for these contradictions. However, some of the differences can perhaps be attributed to two other features of the

observations which are not in agreement. Furukawa and Furukawa did not observe the delayed action of methylammonium chloride which caused a progressive increase in the quantal content in the present experiments. The absence of this observation could be explained if the reduction in the quantal content, which would be expected as a result of the decline in the amplitude of the presynaptic action potential was eliminated by the coincidence of the secondary effect of methylammonium chloride on the presynaptic nerve terminal which leads to an increase in the output of transmitter.

A less contrived explanation is possible on the basis that Furukawa and Furukawa found that the replacement of sodium chloride by methylammonium and ethylammonium chlorides caused an increase in the postsynaptic sensitivity to acetylcholine whereas in the present experiments the mean amplitude of the spontaneous e.p.p.'s was slightly reduced when the sodium substitute was methylammonium and greatly reduced when it was ethylammonium. Furukawa and Furukawa observed an increase in the amplitude of the depolarization of the end-plate region of the toe muscle caused by acetylcholine, when all the sodium chloride in the Ringer solution was replaced by either methylammonium or ethylammonium chlorides. This increase in the depolarization caused by acetylcholine was attributed to an increase in the membrane resistance which was shown to occur when



muscles were exposed to these ions. An increase in the sensitivity to acetylcholine due to an increase in the membrane resistance would be expected if the replacement of all the sodium chloride by either methylammonium or ethylammonium chlorides led to a decrease in the pH of their Ringer. Del Castillo, Nelson and Sanchez (1962) found that the depolarization of the end-plate region of the sartorius muscle bathed by a constant concentration of acetylcholine was greatly enhanced when the pH of the Ringer was decreased from 7 to 4 by the addition of hydrochloric acid. The augmented sensitivity of the muscle to acetylcholine in low pH was again attributed to a marked enhancement of the effective resistance of the muscle, the average values of which fell within the range of the observed variation in the depolarizing action of acetylcholine elicited by similar pH changes. Brooks and Hutter (1962) found that a reduction of the pH from 9.8 to 5.0 increased the membrane resistance 3 fold. They attributed this to a reduction in the permeability of the membrane to chloride ions due to the presence of hydrogen ions. This explanation for the divergence between the present results and those of Furukawa and Furukawa should clearly be tested directly.

## SUMMARY

1) The effect of replacing sodium chloride by methylammonium, ethylammonium and tris-(hydroxymethyl)-amino-methane (tris) chlorides on the quantal content was examined in order to determine whether an increase in the quantal content also occurs when the reduction in the sodium ion concentration is not accompanied by a decrease in the ionic strength of the extracellular fluid.

2) At low calcium concentrations (0.23-0.3 mM) the quantal content was reduced by a factor of approximately 2.5 when one-half of the sodium chloride was replaced by methylammonium chloride. Similar reductions in quantal content also occurred when sodium chloride was replaced by either ethylammonium or tris chloride.

At 1.8 mM calcium, the amplitudes of e.p.p.'s recorded in the presence of tubocurarine, were reduced immediately after the replacement of sodium chloride by methylammonium chloride. During the next 20 min, however, the amplitudes of the e.p.p. progressively increased until they were greater than those recorded originally in the sodium chloride solution.

3) When half of the sodium chloride was replaced by methylammonium chloride, the amplitudes of the miniature e.p.p.'s were only slightly reduced. When the replacement was either ethylammonium or tris chlorides the amplitudes were greatly reduced.

4) The progressive increase in the amplitude of the e.p.p.'s observed at normal calcium concentration in curarized muscle was probably due to an increase in the quantal content. At low calcium concentrations an increase in the quantal content from 2.3 to 10.1 and 2.1 to 6.4 was observed in two experiments, when the muscle was exposed to methylammonium chloride for periods of up to 40 min.

5) At both low and high calcium concentrations the replacement of sucrose by methylammonium, ethylammonium chlorides abolished the approximately five fold increase in the potassium stimulated miniature e.p.p. discharge frequency which occurred when one-half of the sodium chloride was replaced by sucrose. These experiments support the idea that the replacement of sodium chloride by substitutes which maintain the ionic strength does not cause an increase in the probability of release of the individual quantum.

## CHAPTER 7

THE REPLACEMENT OF SODIUM CHLORIDE BY SUBSTITUTES  
WHICH NOT ONLY MAINTAIN THE IONIC STRENGTH  
BUT ALSO THE AMPLITUDE OF THE NERVE  
ACTION POTENTIAL

In chapter 6 it was argued that the decrease in the quantal content which follows the replacement of sodium chloride by ionic substitutes such as methylammonium , ethylammonium and tris chlorides was a consequence of the reduction in the amplitude of the presynaptic action potential. It might be expected therefore that the replacement of sodium chloride by ionic substitutes which maintain the amplitude of the action potential in nerves would cause little alteration in the quantal content. However, it was found that, when sodium chloride was replaced by lithium chloride or hydrazinium monohydrochloride both of which have been shown to maintain the height of the action potential in nerve (Hodgkin and Katz, 1949; Huxley and Stämpfli, 1951; Lorente de No' et al, 1957) the effect on the quantal content in low calcium was extremely variable.

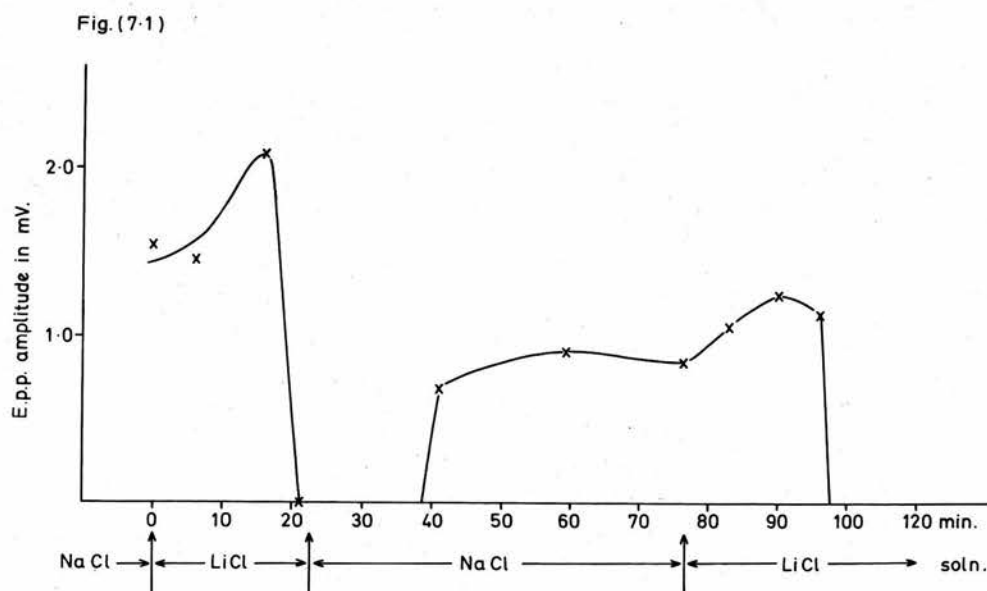


## RESULTS

Lithium chloride

In low calcium. During the first 21 minutes after all the sodium chloride in low calcium Ringer solution had been replaced by lithium chloride in the experiment shown in Fig. (7.1), the amplitude of the end-plate potential recorded intracellularly remained the same for the first 8 minutes, increased in size from 1.5 to 2.1 mV in the next 8 minutes, and then declined extremely rapidly to zero. Immediately after the failure to evoke an e.p.p. the replacement of lithium chloride by sodium chloride caused the e.p.p. to reappear but only after a delay of about 17 minutes. After the e.p.p.'s reappeared they increased rapidly in size for the first four minutes and then more slowly over the next hour; however, they only attained one half of their original amplitude. A second exposure to lithium chloride caused a further increase in the amplitude of the e.p.p. recorded from the same end-plate, from 0.8 to 1.24 mV during the first 15 min after the solution change. Five minutes later, however, the amplitude declined to zero.

In another similar experiment in which the calcium was twice as great ( $1/4$  of the normal) the amplitude of the e.p.p. did not increase but was reduced by a half within five minutes of the exposure to lithium chloride and could no longer be evoked 12 minutes later. As in



**Fig. (7.1)** The effect on the amplitude of the e.p.p. of replacing all the sodium chloride in the extracellular fluid by lithium chloride. The calcium concentration was 0.23 mM.

the previous experiment, e.p.p.'s could not be evoked for some 13 minutes after the sodium chloride was returned and 45 minutes later the e.p.p. had only returned to two-thirds of its original size.

In three experiments where only four-fifths of the sodium chloride was replaced by lithium chloride there was no initial increase in the amplitudes of e.p.p.'s in two of the experiments and the decline to zero occurred within ten minutes. In the two experiments the quantal content was found to be reduced to one-seventh of that in normal sodium nine minutes after the change. In the third experiment, shown in Table (7.1) a continuous record was made of e.p.p.'s evoked by nerve stimuli at 3.2 second intervals. The amplitude of the e.p.p.'s did not steadily decline to zero but fluctuated greatly. However, when the amplitudes of the e.p.p.'s recorded during successive 2 minute periods (about 37) were examined, it was found that the mean amplitude of the e.p.p.'s in each group, apart from the first two minute period in which it was increased, declined steadily to zero. As the mean amplitude of the e.p.p.'s in each successive group declined, the number of failures increased. The mean number of quanta released per nerve impulse predicted by the Poisson distribution from the number of failures in each group agreed with the mean quantal content calculated from the ratio of the mean amplitude of the evoked e.p.p.'s to the mean amplitude of the spontaneous e.p.p.'s

**Table (7.1)** The effect of replacing four-fifths of the sodium chloride by lithium chloride on the amplitude of the evoked and spontaneous e.p.p.'s. The quantal content was calculated either from the ratio of the amplitudes of the evoked and spontaneous e.p.p.'s ( $m_2$ ) or from the ratio of failures to the number of trials ( $m_0$ ) and the miniature e.p.p. discharge frequency at a single end-plate.

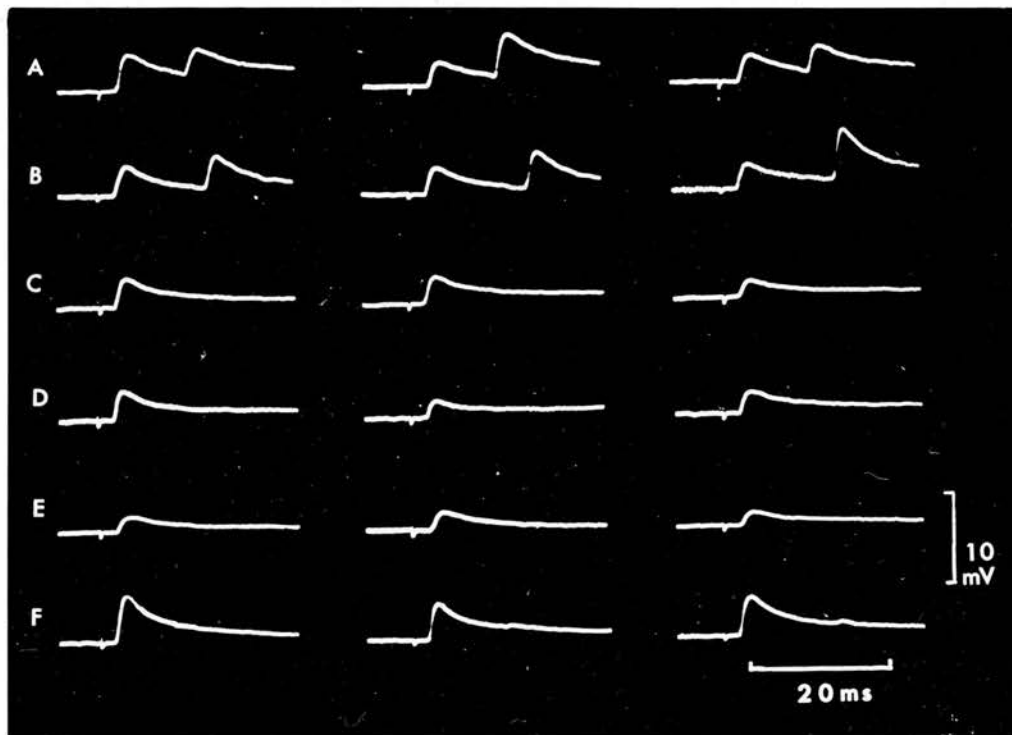
	NaCl	$(\frac{1}{5} \text{ NaCl} + \frac{4}{5} \text{ LiCl})$						
		(0-2)	(2-4)	(4-6)	(6-8)	(8-10)	(10-13)	(13-17)
Time (min)	0							
E.p.p. amplitude mV	0.56	0.72	0.31	0.31	0.30	0.18	0.16	0
M.e.p.p. amplitude mV	0.56	0.55	0.62	0.56	0.60	0.58	0.53	0.59
Quantal content $m_2$	1.0	1.3	0.5	0.5	0.5	0.3	0.3	-
Quantal content $m_0$	0.9	1.0	0.5	0.5	0.5	0.3	0.4	0.05
M.e.p.p. discharge frequency	3.3	7.5	7.6	20.0	41.7	50.9	61.1	31.7



recorded simultaneously during the same two minute period. If the fluctuations in the amplitude of the e.p.p.'s and the failure to evoke an e.p.p. were the result of an intermittent and progressive failure of the action potential to invade the presynaptic nerve endings it would be unlikely that the mean amplitudes of the e.p.p.'s and the number of failures would both indicate the same mean quantal content.

When only a half of the sodium chloride was replaced by lithium chloride the quantal content, in one experiment, was reduced by half after five minutes, from 2.5 to 1.5 quanta and exposure to lithium chloride for another 30 minutes caused only a small further decrease in the quantal content to 0.8 quanta. In another experiment, shown in Fig. (7.2) the more immediate effects of exposure to lithium were examined on another end-plate in the same muscle. It was found that after the replacement of the sodium chloride the mean amplitudes of the e.p.p.'s recorded at 4 second intervals was actually greater during the first three minutes and was only reduced from 3.1 to 2.3 mV after 5 minutes; a further 6 minutes in lithium caused little further change in the amplitude of the e.p.p. The return of sodium chloride caused the amplitude of the e.p.p. to increase very rapidly and within 3 minutes it was 3.46 mV, i.e. 0.44 mV greater than the original size in sodium chloride Ringer solution.





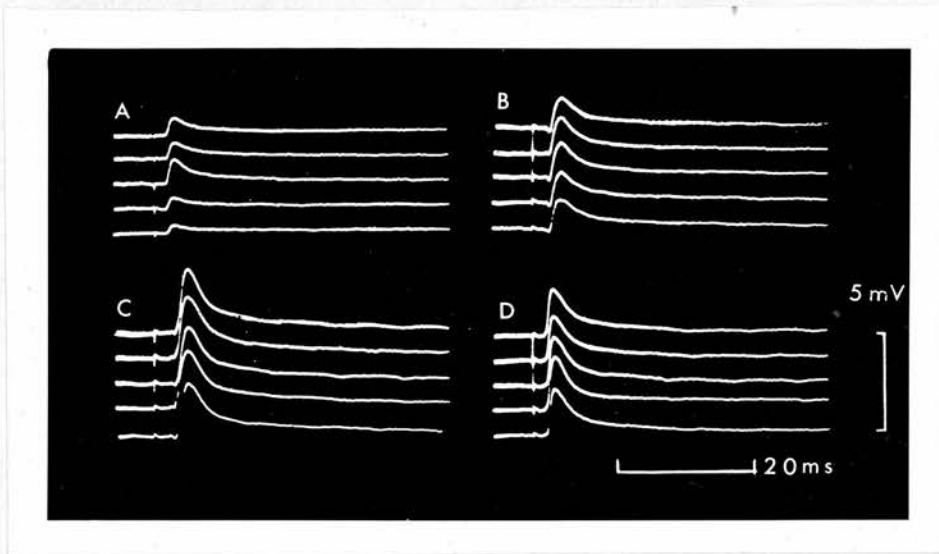
**Fig. (7.2)** The effect of replacing one-half of the sodium chloride by lithium chloride. The calcium concentration was 0.3 mM. The mean amplitude of the e.p.p.'s recorded from the same end-plate was (A) 3.02 mV in normal sodium (B) 3.21 mV 1 min after the replacement (C) 3.10 mV 3 min after the replacement (D) 2.3 mV 5 min after the replacement (E) 2.3mV 1 min after the return to normal sodium conc. and (F) 3.45 mV 3 min after the return to the normal sodium concentration.

The effects of lithium and sodium chloride in the presence of sucrose.

The quantal content should be identical in two solutions containing the same reduced concentrations of sodium or lithium chloride. In the experiment described below it was found that only a small reduction occurred in the amplitude of the e.p.p. when a solution containing one-fifth of sodium chloride and four-fifths sucrose was replaced by another in which the sucrose concentration was the same but containing lithium chloride instead of sodium chloride. (Fig. (7.3) ).

The mean amplitude of the e.p.p.'s increased from 1 mV to 3.9 mV when four-fifths of the sodium chloride in Ringer solution containing 0.225 mM calcium was replaced by sucrose; the increase in the quantal content was from 6 to 50. For a period of 10 minutes after this solution was exchanged for one which the one-fifth sodium was replaced by lithium chloride, the mean amplitude of the e.p.p. was reduced to only 2.7 mV. Two minutes later, however, the amplitude of the e.p.p.'s declined to zero. Only after 7 minutes in the solution containing one-fifth sodium did the e.p.p.'s reappear. The mean amplitude of the e.p.p.'s recorded between 7-10 minutes after the final solution change was 4.3 mV.

In summary, the effect of replacement of sodium chloride by lithium chloride on the quantal content at low



**Fig. (7.3)** The effect of replacing sodium chloride by lithium chloride after 4/5th of the sodium chloride had already been replaced by sucrose. The calcium concentration was 0.23 mM. E.p.p.'s recorded from same end plate in solutions containing 0.23 mM  $\text{Ca}^{2+}$  and various concentrations of NaCl, LiCl and sucrose. A - Normal NaCl (mean amplitude of e.p.p.'s, 1.0 mV). B - 1/5th NaCl and 4/5th sucrose (mean amplitude of e.p.p.'s, 3.9 mV). C - 1/5th LiCl and 4/5th sucrose (mean amplitude of e.p.p.'s, 2.7 mV). D - 1/5th NaCl and 4/5th sucrose (mean amplitude of e.p.p.'s, 4.3 mV).

calcium concentrations was rather variable. In the majority of experiments, 5 to 10 minutes after the sodium chloride was replaced by lithium chloride the quantal content was appreciably reduced and more prolonged exposure to lithium reduced the quantal content to zero. At one end-plate, however, the amplitude of the e.p.p. increased on two separate occasions during the 15 minutes following the replacement of all the sodium chloride by lithium chloride. In experiments where the more immediate effects of lithium chloride were studied, the quantal content in one experiment was increased in the two minute period immediately after  $4/5$ th of the sodium chloride was replaced. In another when only one half of the sodium chloride was replaced the amplitudes of the e.p.p.'s increased during the first 3 minutes after the lithium was introduced. A reduction in quantal content does not invariably occur when sodium chloride is replaced by lithium chloride.

When the quantal content was reduced to zero by exposure to lithium chloride the process which causes the reduction takes a considerable time to reverse when the sodium chloride is returned and recovery is seldom complete. The apparently irreversible nature of the reduction in the quantal content together with two other phenomena described later in the chapter, the acceleration of the miniature e.p.p. frequency and the increase in the

duration of the delay between the nerve stimulus and the e.p.p., suggest that lithium chloride may have a deleterious effect on the presynaptic nerve. For example, if the nerve terminals have little or no capacity to extrude lithium ions, the continued influx of these ions during the passage of action potentials or at rest will cause a progressive rise in the intracellular lithium concentration, a progressive decrease in the intracellular potassium concentration and as a result the nerve terminal will become depolarized. The reduction in the quantal content would occur as a consequence of the reduction in the amplitude of the presynaptic action potential.

#### Normal Calcium.

When the calcium concentration of the extracellular fluid was raised from 0.45 mM to 1.8 mM immediately after nerve stimulation had ceased to evoke an e.p.p. from a muscle bathed in lithium chloride Ringer solution, the e.p.p.'s immediately reappeared and rapidly increased in amplitude until large enough to initiate an action potential. This supports the idea that the failure to release transmitter which developed in lower calcium was due to a reduction in the probability of release of the individual quantum rather than to a failure of the presynaptic action potential to invade the presynaptic nerve terminals. The failure to release transmitter in low calcium when the muscle was bathed in lithium chloride



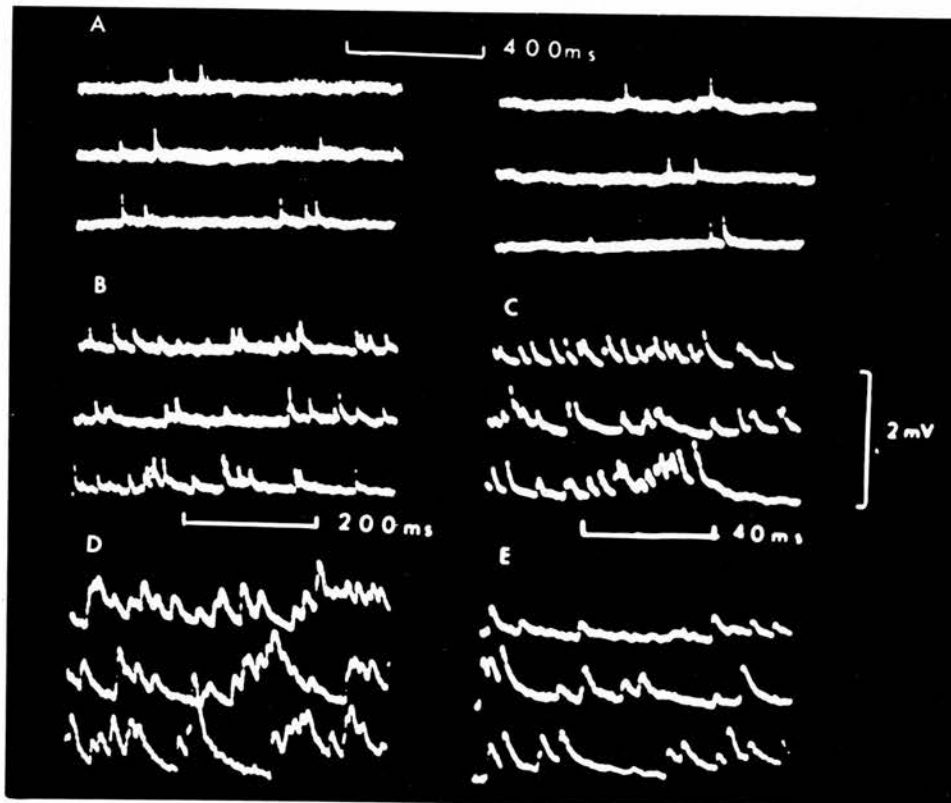
could also be relieved by a brief conditioning tetanus applied to the nerve. This procedure, like an increase in calcium concentration, may also increase the probability of release of the individual quantum.

On the other hand if the reduction in the quantal content is not a consequence of a reduction in the probability of release of the individual quanta but rather the result of a reduction in the amplitude of the presynaptic action potential it is not unreasonable to suppose that a short tetanus (Hubbard and Schmidt, 1962) or an increase in the calcium concentration might increase its amplitude.

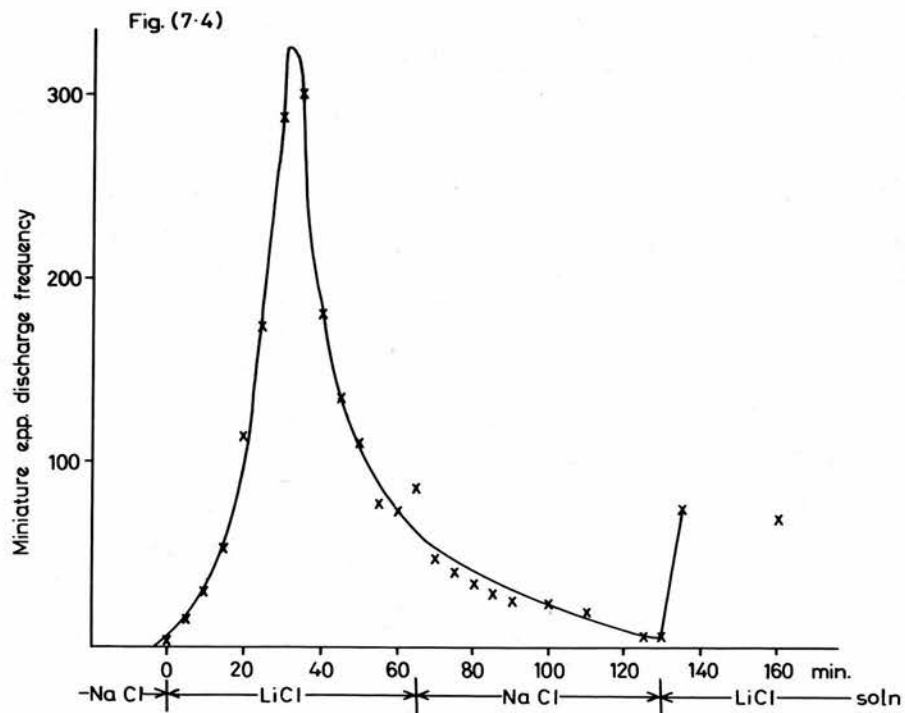
The failure to evoke e.p.p.'s in low calcium Ringer solution in which part of the sodium chloride has been replaced by lithium chloride, very quickly became irreversible. E.p.p.'s could not then be evoked either by an increase in the calcium concentration, by a brief conditioning tetanus or by returning the preparation to sodium chloride Ringer.

#### The effect on the spontaneous e.p.p.'s.

Immediately after the sodium chloride was replaced completely or partially by lithium chloride, the discharge rate of the spontaneous e.p.p.'s began to increase rapidly until a maximum frequency 20 to 200 fold the resting frequency was reached within 10-30 minutes; the recurrence frequency then slowly declined. The increase



**Fig. (7.4)** The effect of replacing sodium chloride by lithium chloride on the miniature e.p.p. discharge frequency. The calcium concentration was 0.45 mM. A - Miniature e.p.p.'s recorded in normal sodium chloride solution (frequency was 2.1/sec). Miniature e.p.p.'s recorded from the same end-plate at intervals after the replacement of sodium chloride by lithium chloride. The frequency was 29.9/sec after 10 min. (B), 114.5/sec after 20 min (C), 282/sec after 35 min (D) and 75/sec after 60 min (E). The oscilloscope sweep speed was identical in C, D and E.



**Fig. (7.5)** The relationship between the miniature e.p.p. discharge frequency and the time after the replacement of sodium chloride by lithium chloride. The results are from the experiment illustrated in Fig. (7.4).

in frequency occurred at low and high calcium concentrations. As shown in Fig. (7.4) and (7.5) the recurrence frequency usually remained high after exposure to lithium chloride for an hour and did not return to the original level for a considerable time after the sodium chloride was replaced. In two experiments, replacement of four-fifths of the sodium chloride by lithium chloride caused very little alteration in the amplitude of the miniature e.p.p.'s. On another occasion replacement of half of the sodium reduced the amplitude of the miniature e.p.p.'s by one quarter.

The effect of lithium chloride on the conduction velocity of the nerve impulse.

In Fig. (7.6) are shown records from two separate experiments in which one half of the sodium chloride in Ringer solution containing 0.4 mM calcium chloride was replaced by lithium chloride. The first two series of records show that the increased amplitude of the e.p.p. which occurred immediately after the sodium chloride was replaced by lithium chloride was accompanied by a small decrease in the delay between the stimulus artifact and the initial rise of the e.p.p. Since the majority of this delay is due to the conduction of the nerve impulse along the nerve, it could be argued that the initial exposure to lithium caused little reduction if not an increase in the speed of conduction of the nerve impulse in the nerve. However, in another experiment illustrated

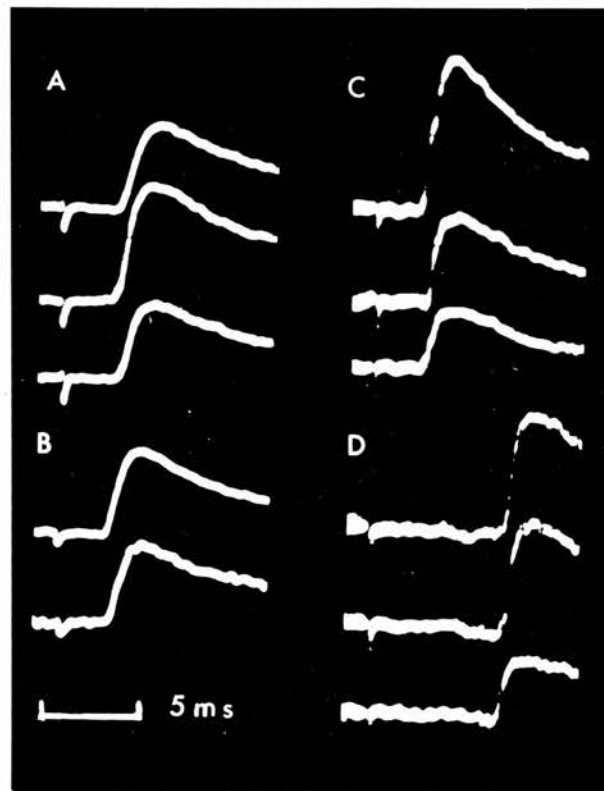


Fig. (7.6) The effect of lithium chloride on the conduction velocity of the nerve impulse. E.p.p.'s recorded at the same end-plate before (A) and 5 min after (B) one half the extracellular sodium chloride was replaced by lithium chloride. C - E.p.p.'s recorded from another end-plate 5 min after one-half of the sodium chloride was replaced by lithium chloride. D - E.p.p.'s recorded from the same end-plate 80 min later. Large e.p.p.'s were selected in order to avoid confusion between evoked and spontaneous e.p.p.'s.



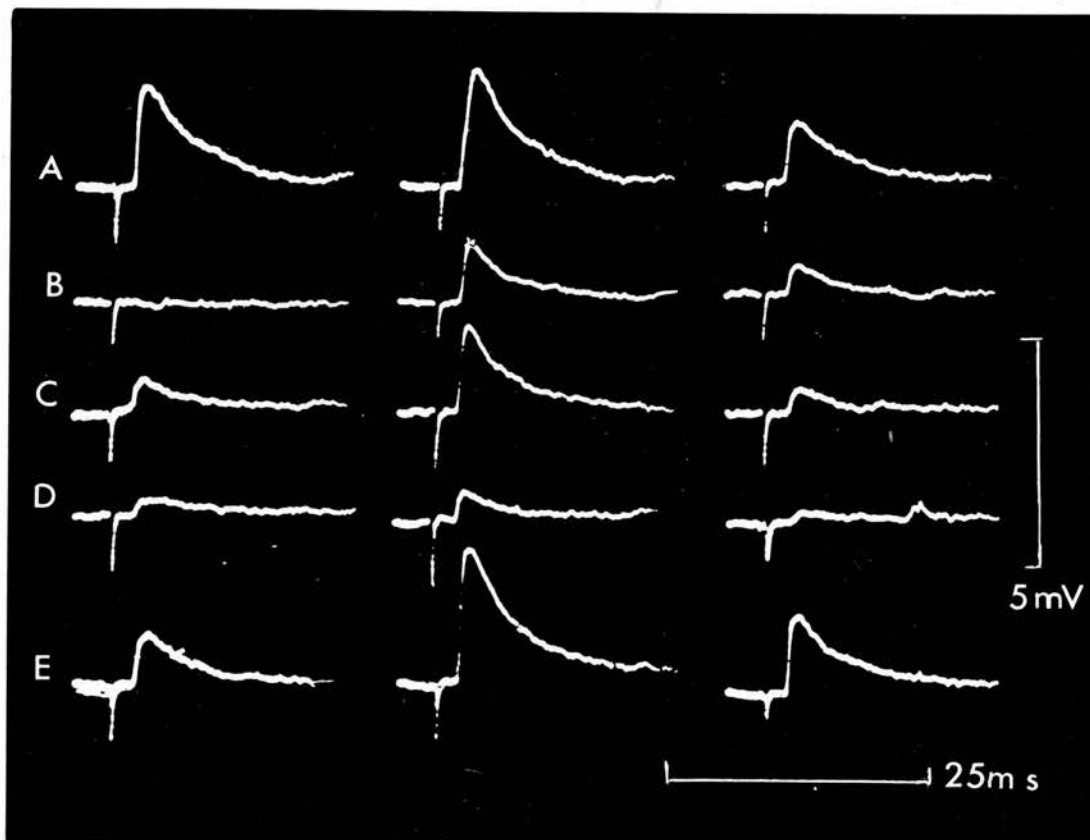
by the third and fourth set of records the reduction in the amplitude of the e.p.p. by a factor of 3 or 4 was accompanied by almost a 2-fold increase in the duration of the delay between the stimulus artifact and the e.p.p. In this experiment the initial records were made during the first five minutes after exposure to lithium chloride and the final records after exposure for a further 80 minutes.

Both the reduction in the quantal content from 4.0 to 0.9 quanta and the decrease in the conduction velocity of nerve impulses in the presynaptic nerve could result from depolarization of the nerve by lithium ions. Depolarization of the nerve would cause a reduction in the amplitude and conduction velocity of the nerve action potential. Regardless of the mechanism involved, it is evident that prolonged exposure to lithium causes undesirable changes in the nerve terminal and that only the changes in the quantal content which occur immediately after the replacement of sodium chloride can be considered relevant. Unfortunately the immediate effects of replacement of sodium chloride by lithium chloride have been found to be rather variable and it is impossible to differentiate between a reduction in the quantal content caused by the presence of lithium ions and a reduction due to a possible depolarization of the nerve terminal.

Comparison between the effects of methylammonium and lithium chlorides.

In chapter 6 it was shown that the replacement of sodium chloride by methylammonium chloride causes a reduction in the quantal content and the reduction was attributed to a decrease in the amplitude of the pre-synaptic action potential. In two experiments described below it was found that the replacement of methylammonium chloride by lithium chloride caused an immediate increase in the quantal content.

In the experiment illustrated in Fig. (7.7) the mean amplitude of the ten e.p.p.'s recorded between 96 and 136 sec after replacement of one half of the sodium chloride by methylammonium chloride, was 0.53 mV, one-fifth of the mean amplitude of the e.p.p. recorded in sodium Ringer solution. The mean amplitude of the e.p.p. recorded 204-244 sec after the exchange of methylammonium chloride for lithium chloride was 0.97 mV i.e. 1.8 times greater than in methylammonium chloride. A further exposure to methylammonium chloride caused an even greater reduction in the mean amplitude of ten e.p.p.'s recorded 40-50 secs. later, to 0.43 mV. The increase in the magnitude of the reduction was most likely an artifact caused by some deterioration of the muscle fibre since the mean amplitude of the e.p.p. recorded after the return to full sodium was only 2 mV instead of the 2.65 mV in the beginning of the experiment.



**Fig. (7.7)** Comparison between the effects of replacing sodium chloride by either methylammonium chloride or lithium chloride on the amplitude of the evoked e.p.p. A - E.p.p.'s recorded in normal sodium chloride solution at 1 every 4 sec; mean amplitude 2.67 mV. E.p.p.'s recorded from the same end-plate (B) 2 min after one-half of the sodium chloride was replaced by methylammonium chloride, mean amplitude 0.52 mV, (C) 3 min after the methylammonium chloride was replaced by lithium chloride, mean amplitude 0.97 mV, (D) 1 min after the lithium chloride was exchanged for methylammonium chloride, mean amplitude 0.43 mV, and finally (E) 7 min after the return of the full concentration of sodium chloride, mean amplitude 2.00 mV. The calcium concentration was 0.3 mM.

In another experiment in which miniature e.p.p.'s and e.p.p.'s were recorded together, the quantal content of the e.p.p.'s recorded from a muscle fibre bathed in a solution in which one-half of the sodium chloride had been replaced by methylammonium chloride was 2.7. Two minutes after the methylammonium chloride was replaced by lithium chloride the mean quantal content of the e.p.p.'s was 4.4. Although the quantal content remained at this level for a further 3 min, more prolonged exposure to lithium chloride for 16 min led to a reduction in the quantal content to 2.2.

Both experiments show that replacement of methylammonium chloride by lithium chloride causes an immediate increase in the quantal content.

#### Hydrazinium.

Before 120 mM isotonic hydrazine monohydrochloride was used as a sodium chloride replacement the pH of 500 ml of solution was brought to 6.8 by the addition of 5 ml of isotonic phosphate buffer and approximately 1.2 ml of isotonic sodium bicarbonate.

In an experiment when the sodium chloride in low calcium Ringer solution was replaced by hydrazine monohydrochloride, the number of stimuli which failed to evoke an e.p.p. increased rapidly until no responses were

observed. At the same time, however, there was a marked increase in the frequency of the miniature e.p.p.'s. The neuromuscular block was temporarily relieved when the calcium concentration in the Ringer solution was increased to its normal value (Fig. 7.8). Evoked e.p.p.'s reappeared and grew rapidly in size until they were large enough to initiate an action potential. Shortly afterwards, however, there was no response to nerve stimuli in spite of the presence of miniature e.p.p.'s. The effect of replacing sodium chloride by hydrazinium mono hydrochloride thus appears to be similar to substitution by lithium chloride.



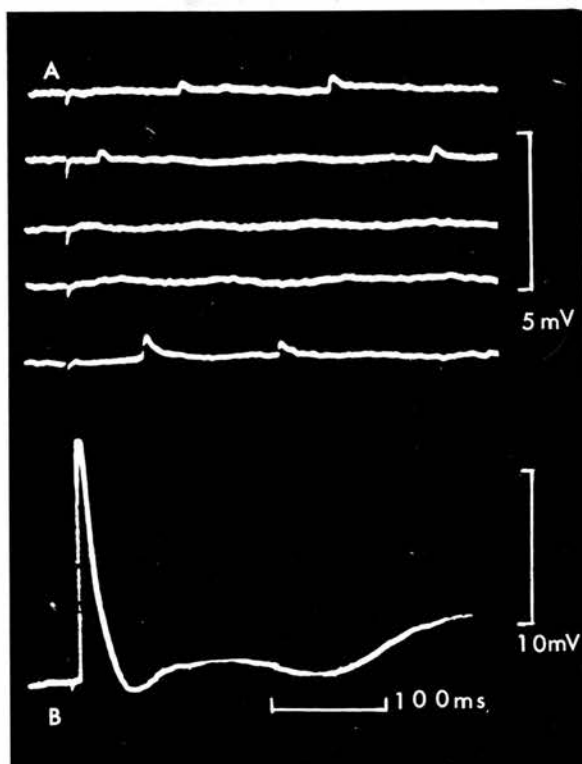


Fig. (7.8) The effect of an increase in the calcium concentration from 0.23 to 1.8 mM on the neuromuscular block which developed after exposure to hydrazinium mono hydrochloride Ringer solution. A - Failure of nerve stimuli to evoke e.p.p.'s 10 min after the sodium chloride was replaced by hydrazinium mono hydrochloride. Note high miniature e.p.p. discharge frequency. B - A large e.p.p. recorded immediately after the calcium concentration was increased to 1.8 mM. Trace distorted by movement artifact.

## DISCUSSION

In chapter 6 it was proposed that a reduction in the quantal content which follows the replacement of sodium chloride by methylammonium, ethylammonium, and tris chlorides was not due to the reduction in the sodium ion concentration in itself but a consequence of a reduction in the amplitude of the presynaptic action potential. If this were the case, no reduction in the quantal content should occur when sodium chloride is replaced by lithium chloride.

Unfortunately the effect of substitution of lithium chloride for sodium chloride on the quantal content has been shown to be extremely variable. There is some doubt, however, as to whether the amplitude of the presynaptic action potential is fully maintained when sodium chloride is replaced by lithium chloride. After prolonged exposure to lithium chloride deleterious changes occur in the motor nerves which can both slow the velocity of conduction of the nerve impulse or render the nerve inexcitable. Immediate changes in the nerve terminal are more difficult to demonstrate but the increase in the miniature e.p.p. discharge frequency suggests that progressive changes might begin very early after the change to lithium chloride.

It would not be unreasonable to suppose that when the extracellular sodium chloride is replaced by lithium

chloride, the nerve terminals become progressively depolarized due to a replacement of intracellular cations by lithium ions which the nerve terminals are unable to extrude. The decline in the amplitude of the presynaptic action potential as a consequence of the depolarization would result in a reduction in the quantal content. It is also possible that the permeability change to lithium ions during the action potential is not as great as that for sodium (cf. Niedergerke and Orkand, 1966) and for this reason the amplitude of the action potential will not be so great.

However, even if the amplitude of the action potential is reduced when lithium is substituted for sodium ions it would be expected that a greater reduction in the quantal content would occur when sodium chloride is replaced by methylammonium chloride. The experiments in which the substitution of lithium ions for methylammonium ions caused an immediate increase in the quantal content are therefore consistent with the idea that the reduction in quantal content in the presence of methylammonium ion is due to a reduction in the amplitude of the presynaptic action potential.

Some of the effects of lithium ions observed have already been reported. Gallego and Lorente de No' (1951) found that although lithium ions restored the action potential in nerves rendered inexcitable in a sodium free

medium, the effect was only temporary and the nerves became once more inexcitable after one hour in the presence of lithium chloride.

On the neuromuscular junction of the frog Okada and Kishida (1961) found that the amplitude of the e.p.p. rapidly decreased to 1/10th of the original size within 50 min of replacing the normal Ringer by lithium chloride Ringer. When Klingman (1965) replaced sodium chloride by lithium chloride in the medium bathing the isolated superior cervical ganglion of the rat, the externally recorded synaptic potential was progressively reduced in amplitude and transmission ultimately failed. The increase in the miniature e.p.p. discharge frequency caused by lithium ions has also been shown to occur in the presence of hydrazinium ions and ammonium ions (Furukawa, Furukawa and Takagi, 1957) both of which share with lithium ions the ability to maintain the amplitude of the presynaptic action potential (Gallego and Lorente de No', 1947 and Lorente de No' et al, 1957). Thirty minutes after sodium chloride was replaced by hydrazinium chloride Koketsu and Nishi (1959) found, as in the present experiments, that e.p.p.'s could not be evoked in the curarized muscle.

The replacement of sodium chloride by glycine and sucrose rather than by methyl- and ethyl- ammonium chloride and by lithium chloride not only causes a reduction in the ionic strength which may explain the increase in quantal content but also causes a reduction

in the extracellular concentration of chloride ions. It is extremely improbable that the reduction in the chloride concentration is responsible for the increased quantal content. In an experiment in which a muscle was bathed in a solution which contained one-eighth of the normal calcium concentration and in which the sodium chloride was replaced by sodium methyl sulphate the quantal content (1.8 quanta) was found to be within the usual range for normal sodium chloride, one-eighth calcium Ringer solution. Furthermore, there was no significant decrease in quantal content when the sodium methyl sulphate was replaced by sodium chloride.



## SUMMARY

- 1) The effect of replacing sodium chloride by lithium chloride on the quantal content was examined on the assumption that lithium chloride will not only maintain the ionic strength but also the amplitude of the presynaptic action potential.
- 2) The effect of replacement of sodium chloride by lithium chloride on the quantal content was extremely variable. In low calcium concentrations the quantal content appeared to be maintained and was occasionally increased in the period immediately after the sodium chloride was exchanged for lithium chloride. In the majority of experiments, however, 5-10 min after the substitution the quantal content was appreciably reduced and a more prolonged exposure reduced the quantal content to zero. It is suggested that the reduction in quantal content occurs not as a consequence of the exchange of sodium ions for lithium ions but the result of a secondary deleterious effect of lithium ions on the nerve terminal.
- 3) Several other effects were observed which were also attributed to the deleterious effect of lithium chloride on the nerve terminals. The reduction in quantal content which occurred after exposure to lithium chloride, was not immediately reversed when sodium chloride was

returned and complete recovery to the original quantal content rarely occurred. Exposure to lithium chloride for longer periods led to nerve block which although at first could be relieved by either an increase in the calcium concentration or a brief period of tetanic stimulation, later became irreversible. Shortly after the exchange of sodium for lithium ions, there was a marked increase in the miniature e.p.p. discharge frequency which reached a maximum of 20-200 times the resting frequency within 10-30 min of the change to lithium chloride Ringer solution. After exposure to a solution in which one-half of the sodium had been replaced by lithium chloride, for 80 min, the duration of the delay between nerve stimulation and the start of the e.p.p. was increased by a factor of about 2.

4) The effect of replacing sodium chloride by hydrazinium mono-hydrochloride appears to be similar to substitution by lithium chloride in that there was a progressive decrease in quantal content to zero and an increase in the miniature e.p.p. discharge frequency. Neuromuscular block was temporarily relieved by an increase in the calcium concentration.

5) When the immediate effect of replacing one-half of the sodium chloride by lithium chloride and methylammonium chloride was compared, the quantal content in the

solution containing lithium chloride was twice as great as in methylammonium chloride. This result is consistent with the idea that the reduction in quantal content in the presence of methylammonium chloride is due to the reduction in the amplitude of the presynaptic action potential.

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